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13. ABSTRACT ***(Maximum 200 Words)***

I have taken two complementary approaches towards characterizing the role of the Est1 telomerase subunit in *S. cerevisiae* telomere replication. The first method utilized a unique set of Cdc13-telomerase fusion proteins; this study led to the proposal that Est1 and Cdc13 (a telomere end-binding protein) are co-mediators of telomerase access. The role for Est1 in telomerase recruitment is strongly supported by the demonstration that telomeres can be stably maintained in the absence of Est1, by fusing Cdc13 directly to the catalytic subunit of telomerase, Est2. These studies further suggest that Est1 has a second role in telomere replication (based on the observation that the Cdc13-Est2 fusion was unable to promote telomere elongation in the absence of Est1). To explore this second role further, I isolated a set of alanine-scan mutations in Est1 that retained association with telomerase but displayed telomere replication defects, and identified an *est1* mutant that displayed phenotypes that were distinct from recruitment defective *est1* alleles. The identification of two distinct classes of mutants, both of which associate with an active telomerase enzyme but which appear to perturb different aspects of Est1 function, supports the idea that Est1 has two roles in telomere replication.

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April 2, 2001

U.S. Army Medical Research and Materiel Command
ATTN: MCMR-RMI-S
504 Scott Street
Fort Detrick, MD 21702-5012

RE: DAMD 17-98-1-8020

Dear Sir or Madam,

The majority of my graduate thesis research has been funded through a Breast Cancer Pre-doctoral Traineeship Award, entitled "Analysis of the Role of *EST1* in Yeast Telomerase". This three-year grant will expire on April 22, 2001. However, my role as principal investigator on this grant effectively ended when I graduated from Baylor College of Medicine in December 2000. During the final months of my thesis research, I revisited some earlier projects; this Final Summary report reflects tying up several "loose ends" and preparing quality data for publication. I am currently in the process of preparing a manuscript based on this work, entitled "Mutational analysis of the *Saccharomyces cerevisiae* Est1 telomerase subunit", for submission to *Genetics*.

Although I have not been actively involved in research since August, experiments with Est1 have continued in our laboratory through the work of another graduate student, Kathleen Buckley. Kathleen's research during the past year solidified the major hypothesis that I was pursuing while on this grant, which proposed that Est1 (a subunit of telomerase) functioned together with Cdc13 (a telomere end binding protein) to mediate telomerase access to the telomere. In support of this model, Kathleen generated and screened a library of *est1* alleles, and identified a particular mutant (*est1-60*) that is an allele-specific suppressor of the telomerase defective *cdc13-2* mutation. This finding provides strong genetic evidence that Est1 and Cdc13 directly interact – a conclusive piece of evidence that was missing from my research. Kathleen's work with the *est1-60* mutant has contributed to a recent publication from our lab. For the past few months, Kathleen has been testing a panel of Est1 mutants (including those generated by me while on this grant) for their ability to bind telomeric DNA. This line of investigation expands on Specific Aim 2 of the original grant proposal, which aimed to elucidate if a putative motif in Est1 contained the determinants for DNA binding. Thus, the remaining funding of this grant has gone directly into research outlined in the original grant proposal: identifying Est1-interacting proteins and delineating the residues involved in DNA binding.

After my last Annual Summary report, I was asked to rewrite the Statement of Work, since my research had markedly departed from the original proposal. I received that review in August (after I had finished my experiments and was beginning to write my thesis), and thus I did not submit a revised SOW at that time. I had originally proposed a set of experiments with Est1, which continue to be long-term goals of research in our lab, but detoured to an extremely exciting and successful project that contributed greatly to the fields' understanding of telomerase recruitment in yeast. I am sure you can appreciate that particular aspect of basic science research.

Lastly, I wish to thank you for supporting my graduate thesis research. I am currently still in the laboratory of Vicki Lundblad, and thus my address, numbers and email remain the same.

Sincerely,

Sara K. Evans, Ph.D.

INTRODUCTION

Telomeres, the nucleoprotein complexes present at the ends of chromosomes, perform an essential function in maintaining chromosomal stability. Telomeres are replicated by the reverse transcriptase, telomerase. This enzyme has received a great deal of attention in the field of cancer research, since there is a strong correlation between the presence of telomerase activity and cellular immortalization: telomerase activity is absent in most somatic cells (which have a limited replicative capacity) but is present in the majority of tumors. This observation has led to the model that reactivation of telomerase is a critical event for tumor progression. It has been suggested that inhibitors of the telomerase enzyme may be employed for therapeutic use against epithelial cancers, including breast cancer. Experimental results indicate that certain reverse transcriptase inhibitors, which target the catalytic subunit of telomerase, may indeed be a successful approach for cancer treatment. Identifying additional components of telomerase may further characterize other potential anti-telomerase targets. Towards this goal, my research proposal focuses on examining the role of a telomerase-associated protein, Est1, in telomere maintenance in the yeast *Saccharomyces cerevisiae*. The combined genetic and biochemical approaches that can be used with a yeast model system allows dissection of the molecular details of telomerase function – an important first step in characterizing potential targets in a mammalian system.

RESEARCH SUMMARY (BODY)

BACKGROUND

Telomeres, the ends of chromosomes, are composed of duplex G-rich repeat sequences that terminate in single strand 3' extensions. Maintenance of both the length and terminal structure of the telomere is crucial for genomic integrity and, consequently, for long-term cell viability. A telomere-specific polymerase, called telomerase, is required for telomere replication in nearly all eukaryotes and thus is a key factor involved in proper telomere length maintenance. The telomerase enzyme is a reverse transcriptase that uses an internal RNA subunit as the template to add nucleotides to the G-rich single strand extension of the telomere. In the budding yeast *S. cerevisiae*, the reverse transcriptase catalytic subunit (Est2) and the template RNA (TLC1) have been identified, as well as two regulatory subunits, Est1 and Est3, which are also required for telomere replication *in vivo* [1-4].

Telomere length maintenance requires not only a functional telomerase enzyme, but also a proficient pathway for mediating enzyme access to the end of the chromosome (for review, see [5]). My research has focused on characterizing the role(s) of two proteins in *S. cerevisiae* that are responsible for mediating access of telomerase to the telomere: Est1 and the telomere end-binding protein, Cdc13. The role for Est1 in telomerase recruitment is strongly supported by the demonstration that telomeres can be stably maintained in the absence of Est1, by fusing Cdc13 directly to the catalytic subunit of telomerase, Est2 [6]. Further work in our lab has shown that a recruitment-defective *cdc13* mutation (*cdc13^{est}*) can be suppressed by specific amino acid changes at residue 444 of Est1, suggesting a direct interaction between the two proteins [7]. Therefore, recruitment of telomerase to the chromosome terminus in the budding yeast depends on an interaction between the single-strand end binding protein, Cdc13, and the telomerase-associated Est1 protein [6,7].

In addition to its role in recruiting telomerase, Est1 may also have a second function that is required for full telomere replication. This hypothesis stems from the observation that although the Cdc13-Est2 fusion stably maintains telomeres in an *est1-Δ* strain, it is not capable of promoting the extensive telomere elongation that occurs in the presence of Est1 [6]. This suggests that Est1 may be required to promote telomere elongation by telomerase once the enzyme has been brought to the

telomere; whether this second function involves the DNA or RNA binding properties attributed to Est1 remains to be determined.

My research during the past year has touched upon four projects – a reflection of tying up "loose ends" to complete my thesis research. Work on three of these projects highlighted several interesting aspects of Est1 function, and I am currently preparing a manuscript based on these findings. Project I of this report summarizes the completion of a mutational analyses that was initiated in Year 1 of this grant; the main goal of this project was to obtain quality data suitable for publication. Project II reports on the continuing analysis of the *est1-42* mutation, which displays the properties predicted for an *est1* mutant defective in the putative second function. Further analysis of the *est1-42* allele has shown that it behaves distinctly from recruitment-defective *est1* mutations, supporting the model that Est1 has two separate functions in telomere replication. Results from Project III indicate that the interaction between Est1 and the catalytic subunit of telomerase relies on the presence of the telomerase RNA. Finally, an additional fourth project attempted to detect a physical association between Est1 and Cdc13 *in vivo*. Although this project was unsuccessful in its outcome, it generated useful reagents that have laid the foundation for further research.

Project I: Genetic and biochemical analysis of a panel of *est1* mutants

Rationale

In the initial analysis of the 28 *est1* alanine-scan mutations presented in Year I, the mutants were expressed as HA_3 tagged versions of the protein. However, the parental *HA_3-EST1* gene was expressed on a plasmid that did not fully complement an *est1-Δ* strain (determined after the mutational analysis was initiated). Furthermore, the efficiency of TLC1 recovery in immunoprecipitation experiments by the HA_3-Est1 protein was typically less than 2%, making this reagent less than ideal for analyzing the biochemical properties of the mutant proteins. A subset of these mutants (those that showed telomere replication defects in the first round) were therefore subcloned into a more functional *EST1*-myc_{18} plasmid, and re-tested for a number of phenotypes, including growth, telomere length, overexpression dominant negative phenotypes and biochemical properties of telomerase association. In addition, three mutations were included in this round to investigate the role of a putative RNA binding motif that was reported to be involved in TLC1 binding [8]. The final genetic and biochemical analysis of these 19 alanine-scan mutations, together with the *est1-Δ19* deletion and the previously partially-characterized *est1-6* and *est1-7* mutations [9], are described in this report.

Results and Discussion

Twelve of the alanine-scan mutants displayed interesting phenotypes that provided insight into Est1 function. These mutants are summarized in Table 1 and are described in more detail below:

A. Identification of mutations defective in the telomerase recruitment function of Est1: Eight mutations conferred moderate to severe telomere replication defective phenotypes, and clustered within a central region of Est1 previously shown to be required for nucleic acid binding *in vitro*. These mutant proteins do not appear to be defective for RNA binding, as they associate with TLC1 at levels comparable to the wild-type Est1 protein. In addition, the majority of the mutations in this class conferred a dominant negative phenotype when overexpressed, suggesting that overexpression of mutant forms of Est1 that are able to interact with telomerase components, may titrate out a limiting factor (or factors) required for telomere replication.
B. The putative RNA recognition motif in Est1 is not required for TLC1 binding: One goal of this mutagenesis project was to determine if mutations in the nucleic acid binding domain were defective for TLC1 association. Strikingly, none of the mutants in this region significantly affected the ability of the mutant Est1 protein to associate with the telomerase RNA, including two mutants (Est1-6 and Est1-7) previously reported to have a substantially reduced association

with the telomerase RNA[1]. In addition, a deletion of 20 amino acids encompassing the putative RNP-1 motif was still capable of associating with TLC1. The fact that a deletion mutation in Est1 that removes a putative RNA binding domain retains association with TLC1 at levels roughly 20% that of wild-type strongly suggests that the region is not required for binding the telomerase RNA. Thus if Est1 binds TLC1 directly, the determinants required for the interaction do not appear to lie within the putative RNP-1 motif. The results presented in this report clearly contradict the results presented by the Futcher laboratory regarding the functionality of the putative RNP-1 motif [8]. The reason for the discrepancy between the data presented here and in Zhou et al. is not clear, but must be attributable to differences in either the strain background or experimental procedure.

C. Identification of mutations that are reduced for telomerase association: Three mutants are defined by their substantially reduced ability to co-immunoprecipitate with telomerase. Unexpectedly, however, these mutants do not display any discernable telomerase-defective phenotypes. This suggests that substantial reductions in the ability of a mutant Est1 protein to associate with the telomerase enzyme can occur, with little negative consequences on telomere replication. These mutants likely define a domain required to stabilize the Est1-TLC1 association; these mutants cluster in a region spanning ~115 amino acids near the amino terminus of the protein (outside of the region required for RNA binding *in vitro*).

D. The *est1-42* mutation displays a telomere replication defective phenotype, but maps outside the DNA/RNA binding domain: This allele confers a telomerase defective phenotype in a sensitizing strain background – the only mutation outside of the region required for DNA and RNA binding *in vitro* to do so. The Est1-42 mutant displays a somewhat reduced association with telomerase, however, other mutants that display a substantially more reduced association with telomerase in immunoprecipitation experiments (the 3 mutants mentioned above) do not have a discernable telomerase defective phenotype, suggesting that the mutant phenotype of Est1-42 can not solely be attributed to a reduced association with the telomerase enzyme. This mutant is the subject of further discussion in Project II of this report.

Future directions for this project

One aim of the original grant proposal was to isolate *est1* mutants defective for DNA binding, since the *in vivo* consequences of a such a defect remain unknown. The *est1* mutants presented above remain to be tested for their DNA binding properties *in vitro*. This goal has been the focus of another student in the lab, Kathleen Buckley: in parallel with a panel of random mutants constructed in the DNA binding domain, Kathleen plans to assay the alanine scan mutants for their *in vitro* DNA binding properties.

Project II: Characterization of a putative loss of second function mutant

Rationale

To explore the proposed second role of Est1, I examined whether any of the *est1* mutants (generated in the site-directed mutational analysis) were also defective in promoting telomere elongation in the presence of the Cdc13-Est2 fusion. In Year 2, I identified that one allele, *est1-42*, that fulfilled this criterion. At the time, the *est1-42* mutant awaited further characterization.

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[1] During the course of this mutational analysis, the Futcher lab reported the existence of an RNA recognition motif (termed RNP-1) within the nucleic acid binding domain that was required for TLC1 binding [8]. In their analysis, mutations in the RNP-1 motif reportedly abolished the interaction with TLC1, while the Est1-6 and Est1-7 mutants substantially reduced this interaction.

Results and Discussion

We have previously proposed that the *est1-47* allele specifically compromises the ability of telomerase to access the end of the chromosome [6]. The behavior of the *est1-42* mutation is very complex, but several observations suggest this mutation is not defective for the same recruitment function of *EST1* that is defined by the *est1-47* allele. First, in contrast to *est1-47*, the *est1-42* mutation was not capable of promoting extensive telomere elongation in the presence of the Cdc13-Est2 fusion protein (shown in the Annual Summary for Year 2). Second, a Cdc13-Est1-42 fusion protein did not substantially elongate telomeres in the presence of an endogenous copy of the *CDC13*$^+$ gene (Figure 1), whereas a Cdc13-Est1-47 fusion protein was equally as effective at lengthening telomeres in a *CDC13*$^+$ strain as the wild-type Cdc13-Est1 fusion (data not shown). These results suggested that whereas the recruitment function of *est1-47* can be completely bypassed by tethering telomerase to the telomere, the defect in the *est1-42* mutation cannot. The reduced ability of Est1-42 to promote telomere elongation in these experiments did not appear to be a consequence of a reduced association with telomerase, as the Est1-38 mutant - which displayed even further reduced telomerase association (Table 1) - was still able to promote substantial telomere elongation in the presence of the Cdc13-Est2 fusion, and was similarly effective in conferring telomere lengthening in *CDC13*$^+$ cells when fused to Cdc13 (data not shown). One possibility consistent with the above experiments is that the *est1-42* and *est1-47* mutations are defective for the same recruitment function, but that the reduced ability of the mutant Est1-42 to associate with telomerase contributes to an overall diminished function of the Est1-42 protein relative to Est1-47. However, this hypothesis is inconsistent with a third observation: an *est1-Δ* strain expressing the *est1-42* mutation displayed an increased telomere length relative to an *est1-Δ* strain expressing the *est1-47* mutation (Table 1). The marked differences in the behavior of the *est1-42* and *est1-47* alleles suggest that these two mutations disrupt separate functions of *EST1*, indicating that *est1-42* may define a role for Est1 in telomere replication that is distinct from telomerase recruitment.

Future directions for this project

The amino acids that are altered in the *est1-42* mutant do not lie within the mapped DNA binding domain. However we cannot exclude the possibility that determinants required for DNA binding also lie elsewhere in the protein, and that *est1-42* is, in fact, defective for DNA binding. Further biochemical analysis of the Est1-42 protein, currently being conducted by Kathleen Buckley, should distinguish between these possibilities and help define the second function of Est1.

Project III: Determining if Est1 and Est2 associate in the absence of the telomerase RNA

Rationale and Experimental Approach

Previous biochemical analysis has shown that Est1 and Est2 co-immunoprecipitate from yeast extracts, however these experiments did not examine the dependency of this interaction on the telomerase RNA (T. Hughes, Ph.D thesis). To answer this question, the ability of the Est2 protein to co-immunoprecipitate with Est1 was analyzed in a *tlc1-Δ* strain. The experimental approach employed a strain where an N-terminal protein A-tagged copy of *EST2* (*ProA-EST2*) was integrated into the endogenous *EST2* locus, and the Est1-myc$_{18}$ protein was expressed on a single copy plasmid and under the native promoter (in an *EST1* strain).

Results and Discussion

The ProA-Est2 protein was immunoprecipitated, and the IP complex was assayed for the presence of Est1-myc$_{18}$ in *TLC1* and *tlc1-Δ* cells by western analysis. Although the Est1-myc$_{18}$ protein could readily be detected in an Est2-ProA IP in the presence of TLC1, it failed to detectably co-

immunoprecipitate with ProA-Est2 in *tlc1-Δ* cells (Figure 2A). This was not due to a gross destabilization of either Est1 or Est2 in the absence of TLC1 (data not shown). These results initially suggested that Est1 and Est2 do not form a stable complex in the absence of TLC1.

One possible mechanism to explain the above observation is that TLC1 functions as a "scaffold" onto which Est1 and Est2 bind; Est1 and Est2 may bind to distinct sites on the TLC1 RNA and be held in proximity like beads on a string. In this model, the bridge between the two proteins is lost in the absence of the telomerase RNA. However, the recent finding that TLC1 may be required for assembly of the telomerase enzyme adds a layer of complexity to this model. The TLC1 RNA has binding sites for Sm proteins, suggesting the telomerase RNA is shuttled out of the nucleus – where it is assembled with telomerase proteins – and then shuttled back in [10]. Therefore, it is possible that in *tlc1-Δ* strains, Est1 and Est2 are never assembled into a complex. To address this caveat, extracts were prepared from *TLC1* cells and incubated either in the presence or absence of RNase during the course of the immunoprecipitation experiment; this method allowed for assembly of the telomerase complex prior to removal of the telomerase RNA. Figure 2B shows that the amount of Est1-myc$_{18}$ present in the ProA-Est2 IP was substantially reduced upon RNase treatment. This was not due to destabilization of the Est1 protein in the absence of TLC1, as Est1 levels were unchanged in RNase-treated versus untreated extracts. Thus, the presence of TLC1 is required for a stable association between Est1 and Est2 proteins. These results, in combination with prior observations that Est1 and Est2 independently associate with TLC1, suggest that Est1 and Est2 may not directly interact, but rather are held in close association largely through their interactions with the telomerase RNA.

Project IV: Can a physical interaction between Cdc13 and Est1 be detected *in vivo*?

Rationale and Experimental Approach

Previous immunoprecipitation experiments in the lab have shown that an interaction between Cdc13 and Est1 can not be detected when both proteins are expressed in single copy and from the native promoter. To determine if overexpression of Est1 could drive a detectable association between the two proteins, I performed immunoprecipitations from cells where the levels of Est1 were greatly increased. Alternatively, the interaction between Cdc13 and Est1 might correlate with the timing of telomere replication/telomerase addition, and thus might be more readily detected in a population of S-phase cells. To test this latter possibility, immunoprecipitations were performed from cells arrested in hydroxyurea (late S-phase) and from cells released from an alpha factor arrest (G1).

Results and Discussion

Neither of the two approaches (increased Est1 expression or cell-cycle synchronization) were successful as a means of detecting an association between Cdc13 and the telomerase enzyme (data not shown). However, cells in G1 did not release from the arrest well, and therefore a large population of S-phase cells was not obtained. Towards the end of this line of experimentation, the Zakian laboratory reported that Est1 and Cdc13 could be co-immunoprecipitated when both were overexpressed in yeast, although this assay was apparently insensitive to the Cdc13^{est} mutant that is proposed to reduce this association [11].

Future directions for this project

It would be advantageous in future endeavors to develop a biochemical assay that is sensitive to recruitment defective mutations in either Est1 or Cdc13. In this regard, whether the interaction between Cdc13 and Est1 is enhanced in S-phase, or in the presence of telomeric DNA, are still interesting aspects of the association that should be pursued further.

Table 1: Summary of the *EST1* mutational analysis

allele number	amino acid substitution	growth in *est1-Δ*	growth in *ku-Δ est1-Δ*	telomere length	o/e dominant negative	association with TLC1	association with telomerase activity
RECRUITMENT DEFECTIVE MUTANTS							
est1-46	R455A,R457A,K458A	+ +	-	mod. short	yes	~wild type	~wild type
est1-47	R485A,K487A,R488A	- / +	-	very short	yes	~wild type	~wild type
est1-49	R499A,E500A	+ +	-	short	yes	~wild type	~wild type
est1-50	R499A	+ +	+	very short	no	~wild type	~wild type
est1-51	F501A	+ +	+	very short	no	~wild type	~wild type
est1-52	D510A,D513A,D514A	- / +	-	very short	yes	~wild type	~wild type
est1-54	F511A	+ +	-	mod. short	yes	~wild type	~wild type
est1-55	D513A	+ / -	-	short	yes	~wild type	~wild type
MUTANTS THAT SHOW THE RNP-1 RNA-BINDING MOTIF IS NOT REQUIRED FOR TLC1 BINDING							
est1-6	F511S	+	-	NT	yes	~wild type	NT
est1-7	D513I	- / +	-	NT	yes	~wild type	NT
est1-Δ19	Δ499-518	- / +	-	very short	NT	reduced (20%)	reduced (10-20%)
MUTANTS WITH REDUCED TELOMERASE ASSOCIATION							
est1-38	R111A,R112A,K113A,R115A	+ +	+ +	sl. short	no	reduced (1%)	reduced (2%)
est1-39	R118A,K122A,K123A	+ +	+ +	sl. short	no	reduced (3%)	reduced (30%)
est1-41	E222A,K223A,R226A	+ +	+ +	wild type	no	reduced (2-8%)	reduced (4%)
MUTANT DEFECTIVE IN PUTATIVE SECOND FUNCTION							
est1-42	D287A,E290A,R291A,R292A	+ +	-	mod. short	no	reduced (10-20%)	reduced (30%)

Scale: ++ > + > +/- > -/+ > -

NT = not tested in this analysis

Growth in *est1-Δ*: *est1* mutations were expressed in single copy under the native *EST1* promoter in an *est1-Δ* haploid strain, and scored for growth at ~75 generations.

Growth in *ku-Δ est1-Δ*: *est1* mutations were expressed in single copy under the native *EST1* promoter in a *yku-Δ est1-Δ* haploid strain, which serves as a sensitizing strain background to more stringently analyze the mutant phenotype. Growth was analyzed following loss of the covering wild-type *EST1* plasmid.

Telomere length: single copy *est1* mutations were expressed in an *est1-Δ* haploid strain and analyzed for telomere length by Southern blot analysis following ~60 generations of growth.

Overexpression dominant negative: *est1* mutations were expressed in high copy (under the strong *ADH* promoter and on a 2μ plasmid) in a *yku-Δ EST1* haploid strain, and were monitored for a reduction in viability.

Association with TLC1: *est1* mutants were expressed in single copy under the native *EST1* promoter in an *est1-Δ* haploid strain, and analyzed for association with the telomerase RNA by co-immunoprecipitation followed by northern analysis.

Association with telomerase activity: Mutant Est1 immunoprecipitates were assayed for the presence of telomerase catalytic activity, by incubation with a telomeric primer substrate, labeled TTP, and the chain terminating ddGTP nucleotide.

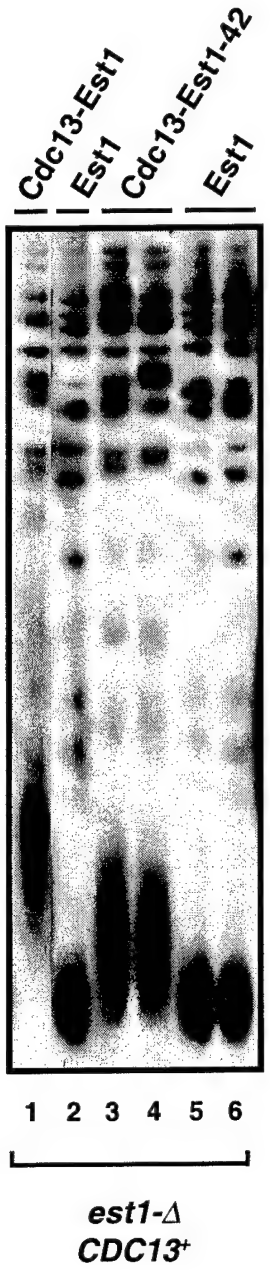

Figure 1: Lengthening properties of the Cdc13-Est1-42 fusion. Plasmids expressing the fusion proteins were introduced into a haploid *est1-Δ CDC13*$^{+}$ strain. Lanes 2, 5 and 6, an *EST* $^{+}$ control strain for wild-type length; lane 1, *est1-Δ CDC13*$^{+}$ /Cdc13-Est1; lanes 3 and 4, *est1-Δ CDC13*$^{+}$/Cdc13-Est1-42; lane 7. DNA was prepared from *est1-Δ* strains ~75 generations following transformation.

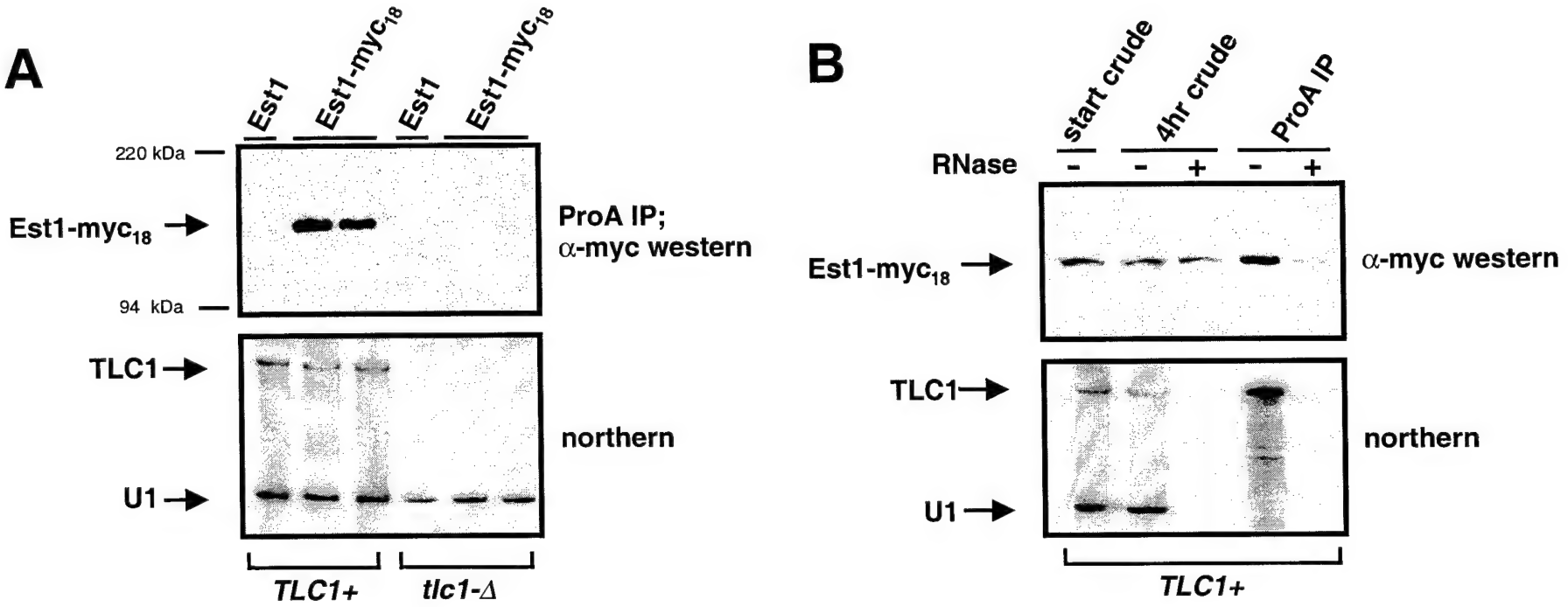

Figure 2: Est1 and Est2 do not co-immunoprecipitate in the absence of the telomerase RNA. (A. top) Plasmids expressing either the tagged Est1-myc$_{18}$ protein or untagged Est1 protein were introduced into a *TLC1* strain carrying an integrated copy of *ProA-EST2* in parallel with a *tlc1-Δ* deletion derivative (harboring a *CEN URA3 TLC1* plasmid). The wild-type *TLC1* plasmid was subsequently evicted by plating on media containing 5-FOA, and cultures were initiated immediately following the loss of the *TLC1* plasmid. Extracts prepared from strains grown in media to maintain selection of the *EST1* plasmid were incubated with IgG Sepharose beads to immunoprecipitate the ProA-Est2 protein, and immunoprecipitates were analyzed by western blotting with the 9E10 anti-myc antibody to detect the presence of the Est1-myc$_{18}$ protein. Duplicate lanes represent two separate isolates analyzed in parallel; this experiment was repeated with identical results. (A, bottom) Northern blot analysis on extracts from samples to demonstrate the absence of the TLC1 RNA in *tlc1-Δ* strains. (B, top) Extract was prepared from a *TLC1* strain carrying an integrated ProA-*EST2* gene and expressing *EST1*-myc$_{18}$ on a plasmid. The extract was divided into two equal samples and both were incubated with IgG Sepharose to immunoprecipitate the ProA-Est2 protein, however one sample was treated with RNase (20µl for four hours during the course of the immunoprecipitation). Aliquots of crude extract and IP pellet were analyzed by western blotting with the 9E10 anti-myc antibody to detect the presence of the Est1-myc$_{18}$ protein. (B, bottom) Northern blot analysis of the samples to demonstrate the absence of detectable TLC1 RNA in RNase-treated preparations. This experiment was repeated with identical results.

KEY RESEARCH ACCOMPLISHMENTS

The following list of key research accomplishments encompasses the three years of research supported by this grant:

- Developed a novel technique to probe the functions of Est1 and Cdc13, two proteins implicated in mediating telomerase access to the chromosome terminus; this analysis involved fusing telomerase components (Est1 and Est2) to the Cdc13 protein. These fusion proteins provided a tool to elucidate the molecular basis for specific defects in the Cdc13 and Est1 proteins, and therefore provided the first detailed insights into the functions of a telomerase holoenzyme component.
  - Fusing Cdc13 to either Est1 or Est2 resulted in a dramatic increase in telomere length, suggesting that fusion of Cdc13 to a telomerase protein greatly increased access of telomerase to the telomere.
  - Certain mutant alleles of either Est1 (generated by a site-directed mutational analysis, described below) or Cdc13 ($Cdc13^{est}$) could be bypassed when present in a fusion, indicating that close physical proximity can bypass telomerase-defective mutations in either protein.
  - Fusing Cdc13 directly to the catalytic subunit of telomerase (Est2) allowed growth and stable telomere maintenance in the complete absence of Est1. This bypass of Est1 function indicated that the primary role for Est1 is to mediate telomerase access to the telomere.
  - However, in the absence of Est1, the Cdc13-Est2 fusion is not capable of promoting the extensive telomere elongation that is observed in the presence of Est1. This suggested that Est1 may have a possible second function – as a positive regulator of telomerase once the enzyme has been brought to the telomere.
  - The telomere lengthening conferred by these fusion proteins was not a consequence of perturbing Cdc13 function as a result of fusing protein sequences to the carboxyl terminus (this was of concern to us since there are telomere lengthening alleles of *CDC13;* [12]). This was addressed primarily by two experiments:
    - fusing the Cdc13 DNA binding domain alone to Est1 ($Est1\text{-}DBD_{Cdc13}$) also results in telomere lengthening, and bypasses the telomerase defective $cdc13^{est}$ mutant.
    - fusing Cdc13 to a catalytically inactive version of Est2 results in wild-type telomere length.
  - These results suggest that telomere length homeostasis is maintained, at least in part, by restricting access of telomerase to chromosome termini, but this limiting situation can be overcome by directly tethering telomerase to the telomere.

- Completed an extensive site directed mutational analysis of the Est1 protein. Twenty-nine *est1* mutants were analyzed for *in vivo* phenotypes, and the corresponding mutant proteins were characterized for their interaction with the telomerase enzyme. This approach yielded several informative mutations:
  - One class of mutations comprise a large group (8) that confer a telomere replication defect but encode an Est1 protein that retains association with an active telomerase complex. The biochemical and genetic properties of these mutants suggest that they are defective in the telomerase recruitment function.
  - Included in this class are mutations in a potential RNA recognition motif (RNP-1). In contrast to previously reported results, we found that certain missense mutations within the RNP-1 sequence do not significantly reduce the ability of Est1 to interact with the telomerase RNA. Furthermore, deletion of the entire RNP-1 motif and surrounding amino acids did not abolish the Est1-TLC1 association, strongly arguing that the putative RRM is not a bona-fide RNA binding motif.
  - A cluster of mutations (3) in the N-terminal region of Est1 that appear to substantially reduce the association with the telomerase RNA were uncovered. These mutants likely define a domain required to stabilize the Est1-TLC1 association.

- The panel of *est1* mutants was screened for those that do not promote telomere elongation by the Cdc13-Est2 fusion, and identified one candidate mutant that may be used to further define the auxiliary role for Est1.
  - Further analysis of this mutant showed that it displayed phenotypes that were distinct from recruitment defective *est1* alleles. The identification of two distinct classes of mutants, both of which associate with an active telomerase enzyme but which appear to perturb different aspects of Est1 function, supports the idea that Est1 has two roles in telomere replication.

- Devised an experimental system to test the hypothesis that Cdc13 mediates telomerase access by recruiting the enzyme to the end of the chromosome, and tentatively assessed that Cdc13 is not sufficient for this recruiting role. This system may be easily manipulated to screen for factors that are required in conjunction with Cdc13 for telomerase recruitment.

- Provided *in vivo* support for the role of Est3 as a subunit of telomerase through the use of Est3-DBD_{Cdc13} fusions, and demonstrated that Est3 does not function in the telomerase recruitment role proposed for Est1.

- Determined that overexpression of dominant negative mutations of *est2* does not affect telomeric silencing.

REPORTABLE OUTCOMES

A. Manuscripts

Full Papers Published in Refereed Journals

1. **Evans, S. K.,** Sistrunk, M. L., Nugent, C. I., and Lundblad, V. (1998) Telomerase, Ku, and telomeric silencing in *Saccharomyces cerevisiae. Chromosoma* **107,** 352-358.
2. **Evans, S. K.,** and Lundblad, V. (1999) Est1 and Cdc13 as comediators of telomerase access. *Science* **286,** 117-120.
3. Hughes, T.R., **Evans, S.K.,** Weilbaecher, R. G., and Lundblad, V. (2000) The Est3 protein is a subunit of yeast telomerase. *Curr Biol.* **10**, 809-12.
4. **Evans, S.K.,** and Lundblad, V. Mutational analysis of the *Saccharomyces cerevisiae* Est1 telomerase subunit. (Manuscript in preparation).

Review articles

1. **Evans, S. K.,** and Lundblad, V. (2000) Positive and negative regulation of telomerase access to the telomere. *J Cell Sci.***113**, 3357-3364.

B. Abstracts and Presentations

Yeast Chromosome Structure, Replication and Segregation — 1998
FASEB Conference
Poster Presentation

Telomeres and Telomerase — 1999
Cold Spring Harbor Conference
Platform Presentation

Analysis of Est1 and Cdc13: Co-mediators of telomerase access
Hal Weintraub Graduate Student Award Presentation 2000
Platform Presentation

Analysis of the Roles of *EST1* and *CDC13* in Telomere Replication
Era of Hope Department of Defense Breast Cancer Research Program Meeting 2000
Poster Presentation

C. Degrees obtained

Ph.D. in Biochemistry 12/2000
Department of Biochemistry and Molecular Biology
Baylor College of Medicine

Thesis Research: Analysis of *EST1* and *CDC13* as regulators of telomerase access in *Saccharomyces cerevisiae*

D. Copies of the above cited manuscripts are provided in the Appendices.

CONCLUSIONS

The research completed under this grant (and supported by further work in our lab) has, importantly, defined the pathway for telomerase recruitment in the budding yeast *S. cerevisiae*: access of telomerase to the chromosome terminus depends on an interaction between the single-strand end binding protein, Cdc13, and the telomerase-associated Est1 subunit. Will this research in yeast translate to human telomere biology? That is the hope of work with model organisms. So far, human homologs of the yeast Est1 and Cdc13 proteins have not been recovered; however, this may be due to incomplete databases or the current limits of search tools designed to identify homologs. The central role of Cdc13 in yeast telomere maintenance [7,12] certainly suggests a human homolog (or functional analog) should exist. Currently, though, there is insufficient information to indicate whether telomerase access at human telomeres will be subject to a positive regulatory mechanism similar to that described for budding yeast. However, a comparison between a particular class of mutation isolated in the catalytic subunits of both human and yeast telomerase provides a potential hint. Friedman and Cech have identified a unique "separation of function" mutation in the reverse transcriptase subunit of yeast telomerase that is defective for telomere replication but retains enzyme activity, which suggests that the catalytic subunit is disrupted for its interaction with a holoenzyme component [13]. A phenotypically similar defect has also been identified in hTERT, the human reverse transcriptase telomerase protein: when this allele of hTERT is transfected into telomerase-deficient cells, telomerase enzyme activity is restored but telomeres are not maintained [14,15]. This suggests that there are factors in human cells that will similarly regulate access of telomerase to the telomere.

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Chromosoma (1998) 107:352–358

CHROMOSOMA

Telomerase, Ku, and telomeric silencing in *Saccharomyces cerevisiae*

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Abstract. Telomeres comprise a specialized chromosome end structure distinct from the standard nucleosomal architecture of the remainder of the genome. Telomere maintenance and chromosome stability require both replication of telomeric sequences by telomerase and telomeric end protection through binding of proteins. We have shown that Cdc13p and the heterodimer Ku are required, along with telomerase, for full telomere function, and we have proposed that Ku and Cdc13p contribute distinct roles in end protection. Ku has recently been shown to exhibit defects in transcriptional repression of telomere-proximal genes, known as telomere position effect (TPE), or telomeric silencing. We investigate here whether alterations in genes involved in the telomerase pathway also exhibit TPE defects and find that deletion or overexpression of *EST1* or *EST2* does not significantly affect telomeric silencing. However, telomeric silencing is derepressed upon overexpression of certain nonfunctional alleles of each. In addition, we determined that overproduction of telomerase pathway components partially alleviates the TPE defect in *hdf1*Δ cells. This indicates that there is genetic crosstalk between these two telomere maintenance pathways, and suggests that overproduction of telomerase pathway components may at least partially compensate for the loss of Ku in maintaining telomeric silencing.

Introduction

Telomeres, as the ends of linear chromosomes, are critical structures that prevent such catastrophic events as end-to-end fusions and chromosome loss via nucleolytic digestion. Telomeres also circumvent loss of sequence in successive replication cycles, known as the "end-replication problem", by employing a specialized telomere-specific DNA polymerase called telomerase. Telomerase is a ribonucleoprotein complex that utilizes its intrinsic RNA subunit as a template for telomere repeat synthesis (for review see Nugent and Lundblad 1998). The telomerase catalytic protein subunit was originally identified in *Euplotes aediculatus* and *Saccharomyces cerevisiae* using biochemical and genetic approaches (Lendvay et al. 1996; Lingner and Cech 1996). The *Euplotes* p123 and the Est2p of *S. cerevisiae* both contain a set of motifs common to known reverse transcriptases; these motifs comprise a protein fold, which forms the reverse transcriptase active site. When potential active site residues are altered in the Est2 protein, telomerase activity is disrupted in vitro and subsequent in vivo telomere replication defects are also exhibited (Lingner et al. 1997a). Since identification of the catalytic component in *Euplotes* and *S. cerevisiae*, this catalytic subunit (called TERT, for telomerase reverse transcriptase) has subsequently been identified in humans, *Schizosaccharomyces pombe* (Kilian et al. 1997; Meyerson et al. 1997; Nakamura et al. 1997; Harrington et al. 1998; Nakayama et al. 1998), *Tetrahymena thermophila* and *Oxytricha trifallix* (Bryan et al. 1998; Collins and Gandhi 1998).

Although the RNA subunit and then TERT protein appear to be sufficient to provide core enzymatic activity in reticulocyte lysates (Weinrich et al. 1997), it is likely that additional components comprise a holoenzyme complex that is required in vivo. These proteins may function to regulate telomerase activity or mediate contact between the core enzyme and the end of the telomere. These additional factors could be components of telomeric chromatin or alternatively could be associated with the core telomerase components to form a telomerase holoenzyme complex. In *Tetrahymena*, the p80 and p95 proteins have been shown to be telomerase associated and are proposed to be involved in DNA-substrate recognition and telomerase-RNA interaction (Collins et al. 1995; Gandhi and Collins 1998). Telomerase-associated mammalian homologs of the *Tetrahymena* p80 protein have also been iden-

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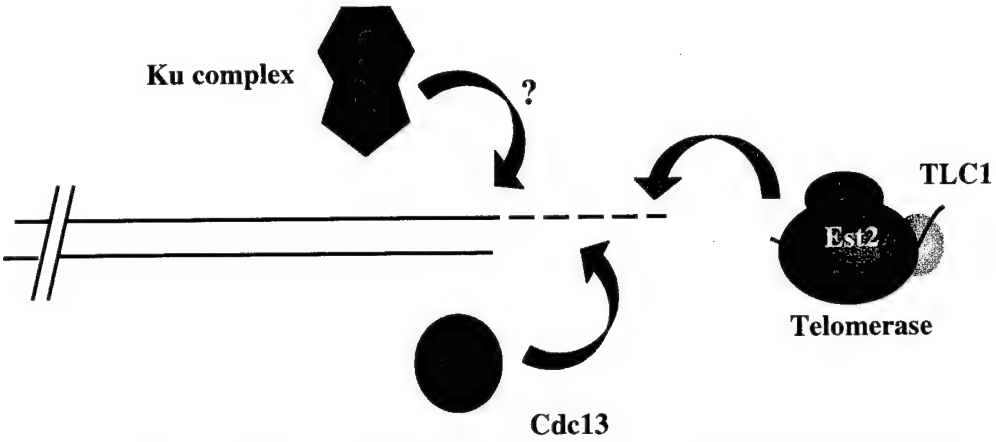

Fig. 1. Three distinct activities are required for telomere function. Ku and Cdc13p have been proposed to have distinct functions at the telomere, perhaps binding the terminal duplex DNA or single-strand G-tail extensions, respectively, at temporally discrete periods in the cell cycle (for review see Bertuch and Lundblad, in press). The telomerase holoenzyme is drawn for purposes of illustration with multiple subunits, although only the templating RNA (TLC1) and catalytic subunits (Est2p) of telomerase have been shown to be components of the enzyme (Singer and Gottschling 1994; Lingner et al. 1997a; Counter et al. 1997)

tified (Harrington et al. 1997; Nakayama et al. 1977). In *S. cerevisiae*, genetic screening for telomere replication mutants has led to the identification of three genes, *EST1, EST3* and *CDC13*, along with *EST2*. Mutations in any of these three genes result in the same phenotype as observed for a defect in the core enzyme: progressive telomere shortening and a gradual decline in cell viability (Lendvay et al. 1996). However, unlike strains defective for either component of the core enzyme (Est2p and TLC1), strains carrying mutations in *EST1, EST3,* and *CDC13* still retain enzyme activity in vitro (Cohn and Blackburn 1995; Lingner et al. 1997b). Est1p binds single-strand G-rich telomeric DNA in vitro, and associates with the telomerase RNA in vivo, suggesting that Est1p may function in bringing the chromosome terminus substrate into the enzyme active site (Lin and Zakian 1995; Steiner et al. 1996; Virta-Pearlman et al. 1996). Cdc13p, as defined by the *cdc13-2^{est}* mutation, also has a role in positive regulation of telomerase (Nugent et al. 1996).

One function vital to maintaining the integrity of chromosome ends, which the cell theoretically could perceive to be DNA breaks, is the protection of these specialized ends from degradation or recombination. In addition to its suggested role in telomerase function, Cdc13p has been proposed to have a second role in maintaining telomere integrity by protecting telomeric termini via end-binding. The *cdc13-1^{ts}* mutation suggests a role for Cdc13p in end protection, because it confers the loss of the telomeric C-rich strand and results in subsequent cell death (Garvik et al. 1995). In addition, Cdc13p binds with high affinity to single-strand telomeric DNA substrates in vitro (Nugent et al. 1996). Since in *S. cerevisiae* the G-rich strand has been shown to exist as a single-strand extension late in S-phase (Wellinger et al. 1993), Cdc13p may function as a cap to telomeric ends at certain stages of the cell cycle.

Another potential player in telomeric end protection is the Ku heterodimer, comprising the *HDF1* and *YKU80* gene products. Ku has been shown previously to bind DNA ends and to mediate nonhomologous end-joining (NHEJ) in both yeast and mammalian cells (reviewed in Jin et al. 1997). Recent evidence suggests that Ku also appears to play an important role in telomeric end protection in yeast. First, absence of either *YKU80* or *HDF1* incurs telomere shortening (Porter et al. 1996; Boulton and Jackson 1998; Nugent et al. 1998). Second, crosslinking experiments suggest Ku80p is physically associated with telomeric chromatin in vivo (Gravel et al. 1998). One hypothesis is that a more discretely defined physical telomeric location for Ku may in fact be the terminal duplex ends, as opposed to a part of internal duplex chromatin (see Bertuch and Lundblad 1998). Finally, further evidence that Ku function is important for establishing or maintaining telomeric chromatin structure is the change in expression of telomere-localized genes in cells lacking Ku function (Boulton and Jackson 1998; Gravel et al. 1998; Laroche et al. 1998; Nugent et al. 1998).

Ku and Cdc13p have been shown to interact genetically: mutations in *YKU80* have been isolated from a screen designed to identify genes that function in parallel with *CDC13* (Nugent et al. 1998). Their roles in end protection, however, are distinct since growth in *cdc13-1^{ts} ku^{-}* cells is greatly reduced compared with cells harboring the individual mutations (Nugent et al. 1998; Polotnianka et al. 1998). This argues that Cdc13p and Ku exhibit two genetically separable activities required for complete telomere function. Growth defects of either *cdc13-1^{ts}* or *ku^{-} (either hdf1*Δ or *yku80*Δ) cells are more severe in cells also lacking functional telomerase (Nugent et al. 1996; Gravel et al. 1998; Nugent et al. 1998), suggesting that Ku and Cdc13p are involved in a telomere maintenance pathway separate from telomerase-mediated telomere replication (Fig. 1).

Although telomere replication by telomerase is a genetically distinct activity from the end-protection functions of Ku and Cdc13p, telomerase function may also be important for maintaining the structure of telomeric ends. In cells overexpressing truncated *TLC1* cDNAs,

telomeres become shorter and telomeric silencing is disrupted (Singer and Gottschling 1994). One explanation for the loss of silencing in cells overexpressing these alleles of *TLC1* is that mutant forms of the RNA titrate away telomerase components, thereby effectively reducing the amount of active telomerase in the cell. This, in turn, would cause shortening of telomeres by preventing telomeric replication. Subsequent attrition of binding sites for silencing proteins may result in loss of telomeric silencing. One prediction of this model is that any strain with short telomeres would have derepressed silencing. An alternative model for the disruption of telomere position effect (TPE) upon overexpression of these truncated alleles of *TLC1* is that they are titrating out chromatin components that are required for telomeric silencing, and that telomere shortening is not a prerequisite to disruption of silencing.

To distinguish between these two models, we determined whether strains deleted in telomerase pathway genes, known to exhibit short telomeres (Lundblad and Szostak 1989; Singer and Gottschling 1994; Lendvay et al. 1996), demonstrate defects in telomeric silencing. We determined that *est1*Δ, *est2*Δ, or *tlc1*Δ exhibit no significant defects in TPE. To ascertain whether the TPE defect upon overexpression of mutant alleles is unique to the telomerase RNA, we examined whether overproduction of other nonfunctional telomerase pathway components would similarly disrupt telomeric silencing. We found that overexpression of certain mutant alleles of *EST1* or *EST2*, but not the wild-type genes, also disrupts telomeric silencing. Finally, we have previously shown that overproduction of telomerase pathway components can suppress a temperature-sensitive growth defect, which displays a phenotypic lag, in *yku80*Δ cells (Nugent et al. 1998). Given this precedent, we therefore wanted to determine whether overproduction of telomerase pathway components could also suppress the silencing defect in *hdf1*Δ cells, and determined that overexpression of *EST1, EST2,* and *TLC1* partially alleviates the defect.

Materials and methods

Yeast strains. The *EST1* and *EST2* genes were disrupted in the haploid silencing strain UCC3505 (*MATa ura3–52 lys2–801 ade2-101 trp1–Δ63 his3–Δ200 leu2–Δ1 ppr::HIS3 adh4::URA3-TEL DIA5–1*) using gene disruptions generated from polymerase chain reaction amplification of the kanMX2 cassette (Singer and Gottschling 1994; Wach et al. 1994). Primer pairs for each gene were designed with 46 bp homology to regions at the start and stop codons of the *EST1* and *EST2* open reading frame. For the telomeric silencing assays shown in Figs. 2 and 4, the strain UCC3505 (Singer and Gottschling 1994) was used. For the sectoring assays shown in Fig. 3, the strain UCC41 (*MATα lys2 his4 trp1Δ ade2 leu2–3,112 ura3–52* with *URA3* and *ADE2* at telomere VII–L) (Gottschling et al. 1990) was used.

Genetic methods. For the growth assays shown in Figs. 2 and 4, serial tenfold dilutions of haploid strains of the desired genotype were spotted onto appropriate medium and incubated at differing temperatures. To ensure that equivalent numbers of cells were compared for each strain, initial cell density was determined using a hemocytometer. For analysis of high-copy suppression of *hdf1*Δ temperature sensitivity of telomeric silencing, at least two transformants from a minimum of two independent transformations were examined for each plasmid/strain combination. This assay was performed prior to manifestation of the temperature-sensitive growth defect of the *hdf1*Δ strain. Yeast transformations were performed using standard genetic techniques. The *hdf1*Δ strains were transformed with: vector alone (pVL399), pVL784 (2μ p*ADH-EST1*), pVL999 (2μ p*ADH-EST2*), or pVL799 (2μ p*ADH-TLC1*). Wild-type cells were transformed with vector control or pJBN155 (2μ p*ADH-SIR4*$_{CT}$). Cells were grown in selective medium and examined at 23° C (after 5 days) and 36° C (after 2.5 days).

For the adenine sectoring assays shown in Fig. 3, the silencing strain was transformed with: vector alone (pVL248 or pVL399), pVL249 (2μ p*ADH-EST1*), pVL306 (2μ p*ADH-est1–7*), pVL999 (2μ p*ADH-EST2*), or pVL1030 (2μ p*ADH-est2–5*) and plated on the appropriate selective medium containing 40 μg/ml adenine. Following another passage of growth on 40 μg/ml adenine plates, cells were plated on media containing 10 μg/ml adenine. Colonies were allowed to grow for 5–7 days at 30° C prior to a 48 h exposure at 4° C to allow full color development.

Results

Absence of telomerase pathway components does not affect TPE

We tested the effect of the disruption of telomerase function on telomeric silencing using two different approaches. The first method examined telomeric silencing in strains deleted in *EST1, EST2* or *TLC1*. Telomeric silencing was measured by assaying the transcriptional activity of a telomere-proximal reporter gene, *URA3* (Gottschling et al. 1990). Cells expressing *URA3* are unable to grow on medium containing the drug 5-FOA (5-fluoro-orotic acid). In wild-type cells, the telomere-proximal *URA3* gene is transcriptionally repressed and cells are able to survive in the presence of 5-FOA (Gottschling et al. 1990). However, when telomeric chromatin is disrupted, the *URA3* gene is expressed, causing lethality on medium containing 5-FOA. In this experiment, we used a silencing strain in which the *URA3* transactivator, *PPR1*, was absent. In a *ppr1*$^-$ strain, the telomeric *URA3* gene is more sensitive to parameters affecting telomeric silencing (Renauld et al. 1993). In this strain background, deletion of either *EST1, EST2* or *TLC1* had no significant effect on TPE, as evidenced by equivalent growth on non-selective (YPD) and 5-FOA-containing medium (Fig. 2). These platings represent cultures that have grown for approximately 40 generations following loss of the gene, yet before the senescence phenotype of these strains is manifested (Singer and Gottschling 1994; Lendvay et al. 1996; Virta-Pearlman et al. 1996). In contrast, overproduction of the C-terminal portion of Sir4p in this experiment completely abolishes telomeric silencing, as previously reported (Cockell et al. 1995).

TPE is disrupted in cells overexpressing certain mutant alleles of telomerase pathway components

A second strategy that we employed to determine whether the disruption of telomerase function could affect TPE was to overproduce particular mutant forms of the Est1

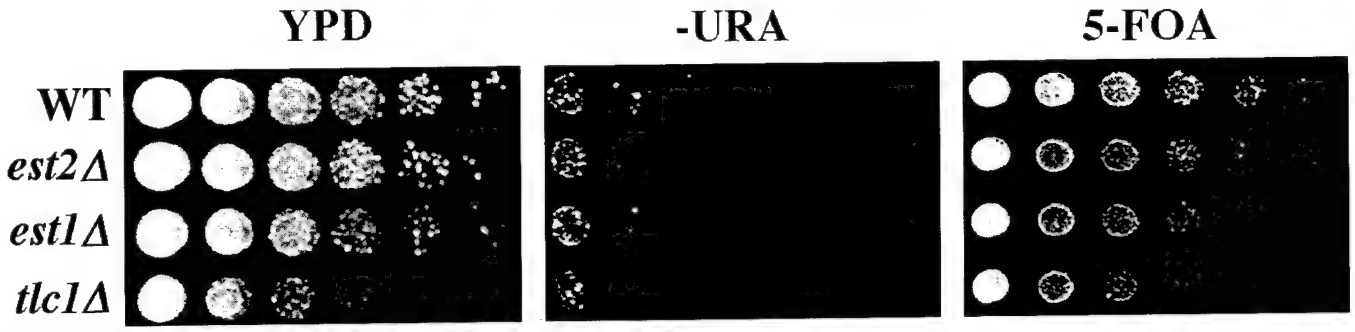

Fig. 2. *EST1, EST2,* and *TLC1* are not required for silencing of telomere-proximal genes. Serial tenfold dilutions of cells from freshly grown wild-type, *est1*Δ, *est2*Δ, or *tlc1*Δ strains were plated on complete medium (YPD) in order to monitor total cells viability, on medium lacking uracil (–URA) to assess the extent of derepression of *URA3* transcription, and one medium containing 5-fluoro-orotic acid (5-FOA) to determine the proportion of cells able to repress *URA3* transcription. Plates were incubated at 30° C for 3 days

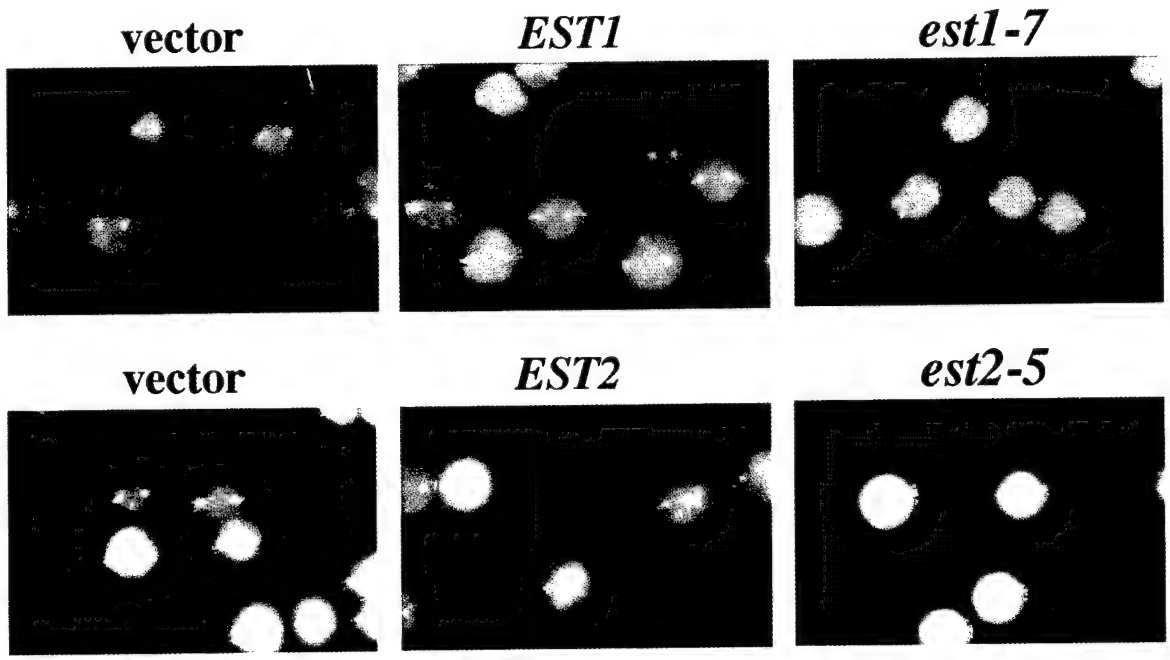

Fig. 3. Overexpression of certain mutant alleles of *EST1* or *EST2*, but not wild-type *EST1* or *EST2*, disrupts telomeric silencing. Wild-type cells harboring plasmids overexpressing wild-type *EST1* or *EST2* or the nonfunctional alleles *est1–7* (Virta-Pearlman et al. 1996) and *est2–5* (Lingner et al. 1997a) or vector control were assayed for the expression state of a telomere-proximal *ADE2* marker. Repression of *ADE2* transcription, reflecting the silencing state, produces red colonies, whereas derepression of *ADE2* transcription, reflecting loss of silencing, produces white colonies

and Est2 proteins. This approach differs from the first experiment in that, although wild-type Est1 or Est2 proteins are still present, the overproduction of nonfunctional forms may be titrating out factors required for telomeric silencing. Thus while the complete absence of the proteins may have insignificant effects on telomeric silencing, it is possible that overproduction of these mutant forms could disrupt telomeric chromatin. As previously stated, overexpression of detective alleles of *TLC1* derepresses telomeric silencing and moderately shortens telomeres, an effect that could be unique to the telomerase RNA component. When particular mutant alleles of either *EST1* or *EST2* are overexpressed in wild-type cells, telomeres also shorten (Virta-Pearlman et al. 1996; Lingner et al. 1997a). We have previously reported that in a *PPR1*$^+$ strain overexpression of certain mutant alleles of *EST1* disrupts telomeric silencing (approximately 500-fold), while overexpression of wild-type *EST1* results in a modest increase in telomeric silencing (Virta-Pearlman et al. 1996). However, Est1 exhibits single-strand telomere binding activity (Virta-Pearlman et al. 1996) and we cannot exclude a role for Est1 as a component of telomeric chromatin. Therefore, we wished to determine whether overproduction of a nonfunctional form of a known protein component of telomerase would have similar effects. We assayed this by taking advantage of a strain with a telomere-proximal *ADE2* marker. In this strain, telomeric silencing is evident by the present of red-sectored colonies (the *ade2*$^-$ phenotype), while disruption of telomeric silencing is manifested as white colonies (the *ADE2*$^+$ phenotype). Figure 3 shows that, like *TLC1* and *EST1*, overexpression of a dominant negative allele of

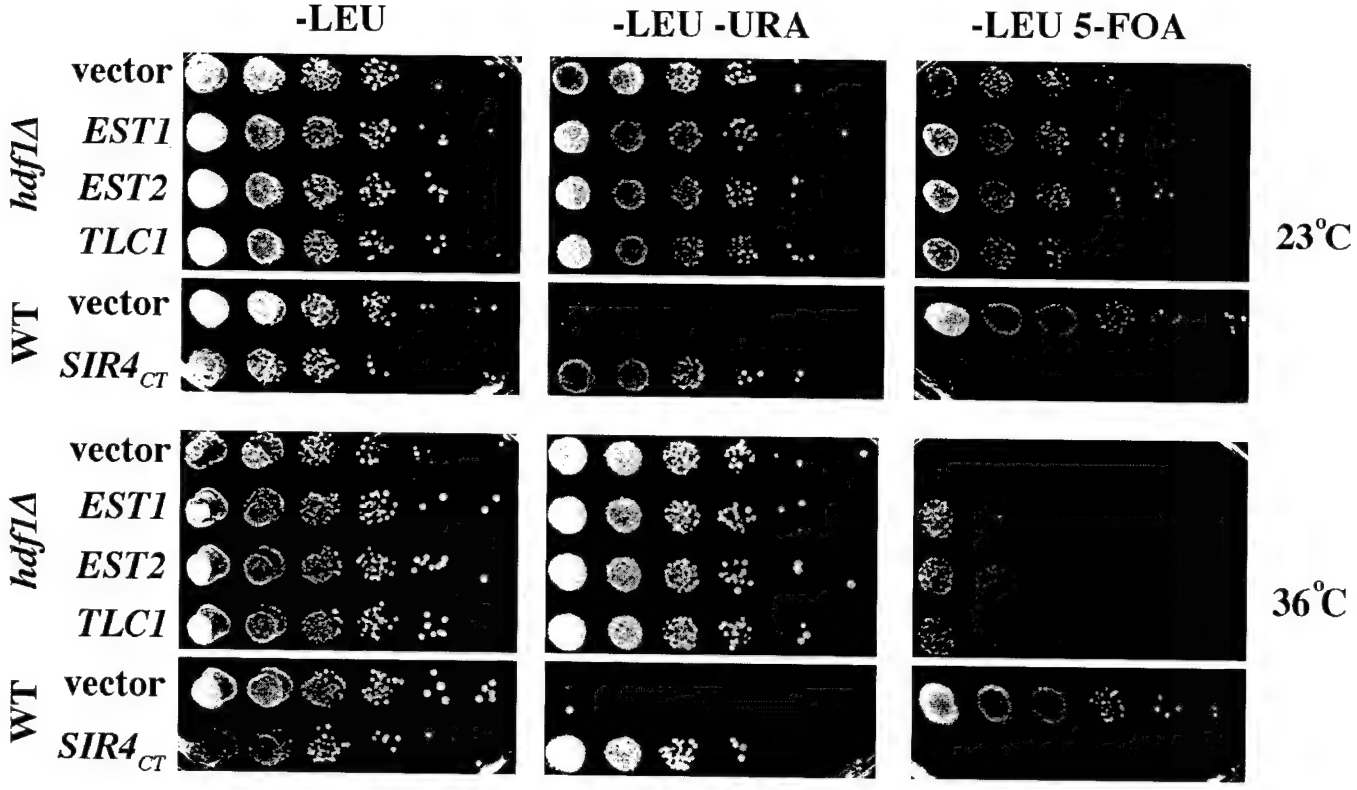

Fig. 4. Overexpression of *EST1, EST2,* and *TLC1* partially alleviates a temperature-sensitive Ku telomeric silencing defect. *hdf1*Δ strains were transformed at 23° C with plasmids overexpressing *EST1, EST2,* or *TLC1,* or a vector control. Wild-type strains were transformed with a plasmid overproducing the C-terminus of Sir4p or its vector control. Serial tenfold dilutions of freshly grown cells were plated on medium lacking leucine to maintain growth in the presence of the respective tester plasmids. Dilutions were grown on plates also lacking uracil (–LEU –URA) to assess the extent of derepression of *URA3* transcription or on medium also containing 5-FOA (–Leu 5-FOA) to determine the proportion of cells able to repress *URA3* transcription. Plates were incubated at either 23° C (5 days) or 36° C (2.5 days)

EST2 also disrupts telomeric silencing, while overexpression of wild-type *EST2* does not. This demonstrates that TPE can be disrupted by overproduction of mutant forms of either of the core catalytic components of telomerase.

Overproduction of telomerase pathway components partially alleviates a Ku TPE defect

Strains deleted for *HDF1* or *YKU80* have been previously shown to have a temperature-sensitive growth phenotype: cells can grow at 23° C but exhibit delayed lethality at 36° C (Feldmann and Winnacker 1993; Barnes and Rio 1997). *ku*$^-$ cells also exhibit a temperature-sensitive defect in expression of a telomere-proximal *URA3* reporter gene (Gravel et al. 1998; Nugent et al. 1998). Since the absence of Ku confers a defect that is seen at high temperatures, this suggests the existence of a thermolabile factor that is partially redundant with Ku at low temperatures. To determine whether altering expression levels of telomerase pathway components affects Ku-dependent TPE activities, we overexpressed *EST1, EST2,* and *TLC1* in *hdf1*Δ cells. Figure 4 shows that *hdf1*Δ cells exhibit a modest TPE defect at 23° C, but at 36° C telomeric silencing is completely abolished, such that cells are unable to grow on 5-FOA, as previously reported (Nugent et al. 1998). However, in a *hdf1*Δ strain overexpressing *EST1, EST2,* or *TLC1,* the temperature-sensitive TPE defect is partially alleviated.

Discussion

The question of whether the disruption of telomerase function would lead to alterations in telomeric chromatin was assayed utilizing two separate approaches. First, we tested the ability of cells to repress *URA3* transcription in the complete absence of the Est1 and Est2 proteins. In our strain background, we did not observe a noticeable defect in telomeric silencing upon deletion of *EST1, EST2* or *TLC1.* This is in contrast to deletion of known chromatin components such as the Sir proteins, where silencing is completely abolished (Aparicio et al. 1991). This suggests that telomerase is not a substantial component of telomeric chromatin, and that the physical presence of the telomerase enzyme is not required to maintain telomeric silencing. We cannot exclude the possibility that there would be long-term effects of the loss of telomerase on silencing, but we have not to addressed this issue because of the decrease in viability in late generation *est/tlc1* cultures.

Second, we tested the effects on telomeric chromatin of overexpression of both wild-type and nonfunctional al-

leles of *EST1* and *EST2*. Overproduction of mutant forms of these proteins in wild-type strains disrupted silencing, whereas overproduction of wild-type proteins did not have a significant effect. There are two possible explanations for this. One is that overproduction of mutant forms of the proteins titrates out other telomerase components that are required for telomerase activity, causing telomere shortening and disruption of telomeric chromatin. However, we think this explanation is unlikely since the complete absence of telomerase components, which also causes telomere shortening (Lendvay et al. 1996; Virta-Pearlman et al. 1996), does not lead to the same phenotype. Instead, a more likely explanation is that overproduction of mutant forms of telomerase components titrates out some factor(s) that is required for telomeric silencing. Candidate factors include Ku itself, or possibly one of the Sir proteins. One way to address which factor is titrated is to determine whether the effects of overexpression of these nonfunctional alleles are telomere specific, or have global consequences in silencing at other loci. Another informative experiment would be to determine whether there is a synergistic TPE defect upon overexpressing the mutant *est* alleles in *ku*$^-$ cells: if the factor being titrated is not Ku, then one would expect a more severe defect upon overexpression of *est1* or *est2* because now both Ku and the candidate silencing factor would be absent from the telomere. One alternative explanation for the TPE defect in cells overexpressing dominant negative alleles of *EST1* and *EST2* may be that they are indirectly affecting TPE by titrating away Ku or some other factor important for telomeric clustering (Laroche et al. 1998).

Although it appears that the telomerase pathway components *EST1, EST2* and *TLC1* are not required directly for telomeric silencing, we questioned whether overproduction of these factors could compensate for the TPE defect observed in *hdf1*Δ strains. To circumvent the possibility that overexpression of *EST/TLC1* genes partially alleviates the temperature-sensitive TPE defect merely by relieving the growth defect seen at 36° C, we assayed repression of TPE at a time point prior to the manifestation of the growth defect. The demonstration that overexpression of these telomerase pathway genes alleviates the Ku silencing defect suggests that there is genetic crosstalk between these two telomere maintenance pathways. Perhaps overexpression of these genes subtly alters telomere structure, or alters the ratio of one type of end structure to another, thereby allowing differential recruitment of particular factors.

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Science

Est1 and Cdc13 as Comediators of Telomerase Access

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Cdc13 and Est1 are single-strand telomeric DNA binding proteins that contribute to telomere replication in the yeast *Saccharomyces cerevisiae*. Here it is shown that fusion of Cdc13 to the telomerase-associated Est1 protein results in greatly elongated telomeres. Fusion proteins consisting of mutant versions of Cdc13 or Est1 confer similar telomere elongation, indicating that close physical proximity can bypass telomerase-defective mutations in either protein. Fusing Cdc13 directly to the catalytic core of telomerase allows stable telomere maintenance in the absence of Est1, consistent with a role for Est1 in mediating telomerase access. Telomere length homeostasis therefore is maintained in part by restricting access of telomerase to chromosome termini, but this limiting situation can be overcome by directly tethering telomerase to the telomere.

In most species, telomeres are composed of G-rich repetitive sequences that are elongated by telomerase (*1*). Several factors govern the balance between sequence addition and loss to maintain telomeres at a stable length, including positive and negative regulation of telomerase access to the chromosome terminus (*2–4*). In *S. cerevisiae*, five genes are required for the telomerase pathway (*4–7*). *TLC1* and *EST2* encode the RNA and reverse transcriptase subunits of telomerase, respectively, and as expected for subunits that are essential for catalysis, telomerase activity is absent in extracts from strains defective in *EST2* or *TLC1*(*7–9*). In contrast, mutations in *EST1*, *EST3*, and *CDC13* do not eliminate enzyme activity in vitro (*9*, *10*), despite the fact that strains carrying mutations in any of these three genes have the same severe telomere replication defect as *est2*-Δ or *tlc1*-Δ strains (*6*, *10*).

Both Cdc13 and Est1 bind single-strand telomeric DNA (*4*, *11*, *12*), although they make separate contributions to telomere replication and stability. Est1 is required solely for the telomerase pathway (*11*), whereas Cdc13 has an essential function at the telomere, presumably in protecting the end of the chromosome (*13*), as well as a role in telomere replication (*4*). This latter activity was revealed by a telomerase-defective allele of Cdc13, called $cdc13^{est}$ [originally named *est4* (*6*)], leading to the proposal that Cdc13, like Est1, mediates telomerase access (*4*). The two proteins also display different biochemical properties. Est1, but not Cdc13, requires a free single-strand 3′ terminus for DNA binding and binds telomeric DNA with a 500-fold reduced affinity compared with Cdc13 (*4*, *11*). In addition, Est1 is associated with telomerase, whereas Cdc13 does not exhibit a detectable interaction with the enzyme (*14*).

These results suggest that telomerase is recruited to the telomere due to a direct (but weak) protein-protein interaction between Cdc13 and the enzyme, and the telomere shortening in the $cdc13^{est}$ mutant strain is due to a further reduction in this interaction. This model predicts that increasing the association between Cdc13 and telomerase would increase telomere length. To test this, we examined the consequences of fusing Cdc13 to the telomerase-associated protein Est1 (*15*). Introduction of the gene encoding this Cdc13-Est1 fusion, present on a single-copy plasmid and expressed by the *CDC13* promoter, into a $CDC13^{+}$ strain resulted in substantial telomere elongation (Fig. 1A, lanes 2

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and 3). This effect was dependent on functional telomerase, because telomeres were not elongated when the fusion was introduced into an *est2*-Δ strain (Fig. 1A, lanes 14 to 16). Telomere elongation was even more pronounced in a *cdc13*-Δ strain, in which telomere length increased by ~800 base pairs after ~100 generations of growth (Fig. 1A, lanes 4 and 5); continued propagation resulted in even further telomere lengthening (*16*).

Several experiments indicated that both Cdc13 and Est1 retained function in the context of the fusion. First, the essential function of *CDC13* was fully complemented by the fusion protein (Fig. 1B). The Cdc13-Est1 fusion also complemented the senescence phenotype of an *est1*-Δ strain (Fig. 1C). This was not due to a general bypass of the telomerase pathway, because this same fusion did not complement *est2*-Δ or *est3*-Δ strains (Fig. 1C). The complementation behavior of the fusion protein indicates that Est1 and Cdc13 normally function in temporal and physical proximity in their respective roles in telomere replication. Telomerase also has been shown to coimmunoprecipitate with Est1 but not with Cdc13 (Fig. 1D) (*14*). The Cdc13-Est1 fusion protein coimmunoprecipitated the RNA subunit of the telomerase complex (Fig. 1D) and enzyme activity (*17*), indicating that telomerase is associated with the fusion protein.

These results suggest that the proposed recruitment function of Cdc13 can be enhanced by fusing it to a telomerase component, and predict that the telomerase-defective *cdc13^{est}* mutation would be bypassed in a fusion. Consistent with this prediction, a fusion containing the mutant Cdc13^{est} protein behaved indistinguishably from the wild-type (WT) fusion: Telomere elongation occurred to the same degree in both *CDC13*$^+$ and *cdc13*-Δ strains (Fig. 1A, compare lanes 6 to 9 and 2 to 5), and no senescence was observed when the Cdc13^{est}-Est1 fusion was introduced into a *cdc13*-Δ strain (*17*). Similar results were observed in a reciprocal experiment with a mutant allele of *EST1* (*est1-47*); this mutation disrupts telomere replication

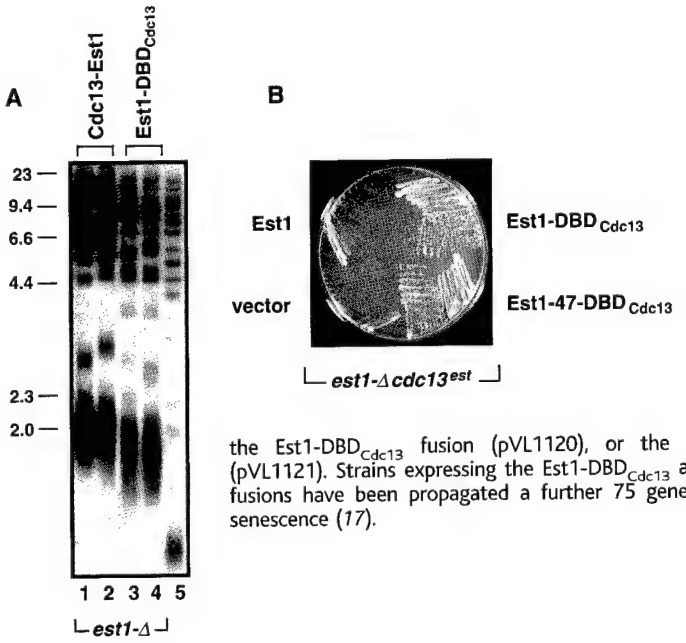

Fig. 2. The DNA binding domain of Cdc13 is sufficient to deliver telomerase to the telomere. **(A)** Telomere length after ~100 generations of growth. Lanes 1 and 2, *est1*-Δ/pVL1091; lanes 3 and 4, *est1*-Δ/pVL1120 (expressing the Est1-DBD$_{Cdc13}$ fusion); lane 5, *EST1*$^+$ control. **(B)** Growth after ~50 generations of an *est1*-Δ *cdc3^{est}* strain with plasmids expressing the Est1 protein (pVL499), the Est1-DBD$_{Cdc13}$ fusion (pVL1120), or the Est1-47-DBD$_{Cdc13}$ fusion (pVL1121). Strains expressing the Est1-DBD$_{Cdc13}$ and the Est1-47-DBD$_{Cdc13}$ fusions have been propagated a further 75 generations with no signs of senescence (*17*).

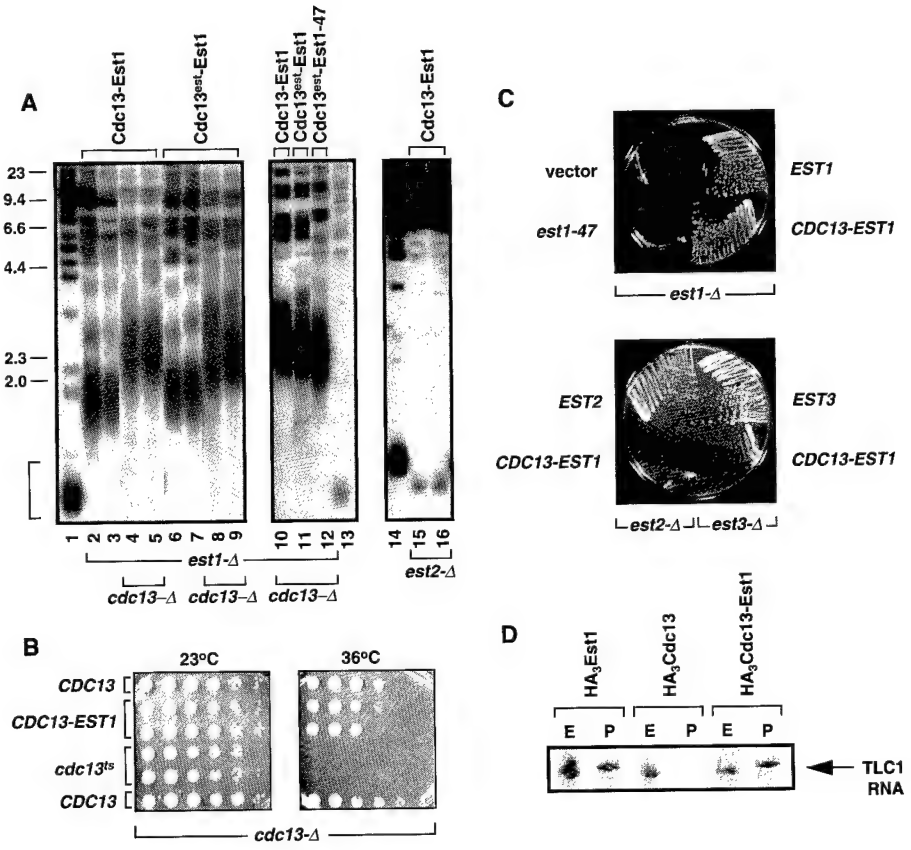

Fig. 1. The Cdc13-Est1 fusion confers telomere elongation. **(A)** Telomere Southern (DNA) blots were performed as in (*6*). The bracket indicates a heterogeneous telomeric band that represents about two-thirds of the telomeres in this strain background. Cultures for lanes 1 to 13 were grown for ~100 generations before DNA preparation. Lanes 1, 13, and 14, *CDC13*$^+$ *EST*$^+$ control strain; lanes 2 to 5, 10, 15, and 16, pVL1091 (expressing the Cdc13-Est1 fusion); lanes 6 to 9 and 11, pVL1092 (Cdc13^{est}-Est1); lane 12, pVL1098 (Cdc13^{est}-Est1-47); the relevant genotypes of the strains are indicated. Plasmids were introduced into strains deleted for *CDC13* for this and subsequent figures by first transforming into a *cdc13*-Δ/pVL438 (*CDC13*$^+$ *URA3*$^+$) strain followed by subsequent eviction of pVL438 by plating on media containing 5-fluoro-orotic acid. Molecular sizes are indicated on the left (in kilobase pairs). **(B)** *cdc13*-Δ strains containing pVL648 (*CDC13*$^+$), pVL1091, or pVL762 (*cdc13^{ts}*) (*13*) were grown at 23°C, and equivalent numbers of cells, as serial 10-fold dilutions, were plated at 23° and 36°C. **(C)** Growth after ~50 generations of *est1*-Δ, *est2*−Δ , or *est3*-Δ strains, with single-copy plasmids bearing the *CDC13-EST1* fusion gene or the appropriate WT *EST* gene (each under their native promoter). **(D)** Immunoprecipitation from extracts prepared from strains expressing proteins with a triple hemagglutinin epitope (HA$_3$) introduced at the NH$_2$-terminus: HA$_3$Est1 (pVL1106), HA$_3$Cdc13 (pVL841), or HA$_3$Cdc13-Est1 (pVL1102), followed by detection of the telomerase RNA (TLC1) levels in the extract (E) and immunoprecipitates (P) by Northern (RNA) blotting (*27*).

(Fig. 1C), although the mutant Est1 protein still physically associates with telomerase (*18*), suggesting a defect in the same telomerase-accessing function that is altered by the *cdc13*^{est} allele. This defect was bypassed when the Est1-47 protein was fused to Cdc13. In fact, the double-mutant fusion protein (Cdc13^{est} fused to Est1-47) conferred the same degree of telomere elongation as the WT fusion protein, even in a strain deleted for both *est1*-Δ and *cdc13*-Δ (Fig. 1A, lane 12); moreover, this strain did not exhibit senescence (*17*). The ability of the double-mutant fusion to complement a *cdc13*-Δ *est1*-Δ strain indicates that the fusion is acting as a dimeric molecule that bridges telomerase and the telomere.

One alternative interpretation of our data is that telomere elongation is due to perturbation of chromatin structure, rather than to increased access of telomerase to the telomere. In particular, telomere lengthening could be a secondary consequence of altered Cdc13 function, because mutations in *CDC13* have been identified that increase telomere length (*19*, *20*). However, these recessive alleles of *CDC13* have a set of genetic and biochemical features that distinguish them from the gain-of-function properties of the Cdc13-Est1 fusion (*17*, *20*). In addition, fusion of several unrelated protein sequences, or an inactive telomerase subunit (see below), to the COOH-terminus of either the WT Cdc13 protein or the mutant Cdc13^{est} protein does not increase telomere length (*17*, *21*). The most direct argument against this alternative interpretation is the result of an experiment in which we examined the behavior of a fusion in which only the high-affinity DNA binding domain of Cdc13 (DBD_{Cdc13}) was fused to Est1 (*15*). This experiment was based on our previous demonstration that DBD_{Cdc13} can be expressed as a stable, functional subdomain (*22*) and therefore could be used as an alternative means of directing Est1 to the telomere with high efficiency, while leaving the full-length Cdc13 protein intact. As predicted, the Est1-DBD_{Cdc13} fusion conferred extensive telomere lengthening in a *CDC13*$^{+}$ strain (Fig. 2A) and bypassed senescence of a *cdc13*^{est} strain (Fig. 2B). Furthermore, fusion of the defective Est1-47 protein to DBD_{Cdc13} bypassed both *est1*-Δ and *cdc13*^{est} mutations (Fig. 2B), even though the *est1-47* allele fails to complement either mutation (Fig. 1C) (*17*). Expression of either Est1 or DBD_{Cdc13} had no effect on telomere length or viability in *CDC13*$^{+}$ or *cdc13*^{est} strains, and the Est1-DBD_{Cdc13} fusion failed to rescue the inviability of a *cdc13*-Δ strain (Fig. 2A) (*17*). Thus, the telomere lengthening properties of these fusions are likely to be a consequence of delivery of telomerase to the telomere, rather than a perturbation of Cdc13 function.

We next fused Cdc13 directly to Est2, the catalytic subunit of telomerase (*8*). The Cdc13-Est2 fusion (*15*) resulted in telomere lengthening to levels comparable to that of the Cdc13-Est1 fusion (Fig. 3A, lanes 3 and 4). The fusion complemented *cdc13*-Δ and *est2*-Δ null mutations, and telomere elongation occurred to the same degree in *est2*-Δ and *EST2*$^{+}$ strains (*17*). A Cdc13-$Est2_{D670A}$ fusion, containing an Asp to Ala mutation at position 670 in the active site of Est2 (*8*), did not confer extensive telomere elongation but instead maintained telomere length at WT levels in a *cdc13*-Δ *EST2*$^{+}$ strain (Fig. 3A, lanes 5 and 6) (*23*), showing that telomere elongation is only observed when a catalytically active version of telomerase is tethered to the telomere. Strikingly, the Cdc13-Est2 fusion allowed cell growth in the complete absence of Est1 function, because an *est1*-Δ strain carrying this fusion was viable for more than 250 generations (Fig. 3B) (*24*). Long-term propagation in the absence of Est1 was not due to a previously described alternative pathway that can maintain telomeres in the absence of telomerase function (*6*, *25*): Telomeres in an *est1*-Δ strain carrying the Cdc13-Est2 fusion were stably maintained at a length slightly below that of WT telomere length (Fig. 3C), with none of the striking changes in telomere structure that characterize the alternative pathway (*6*, *25*). The ability of the Cdc13-Est2 fusion to maintain an *est1*-Δ strain required tethering of a functional telomerase, because an *est1*-Δ strain carrying the Cdc13-$Est2_{D670A}$ fusion exhibited senescence (Fig. 3B). This supports the hypothesis that a critical function of the Est1 protein is to mediate access of telomerase to the telomere. Notably, neither the Cdc13-Est1 fusion nor the Cdc13-Est2 fusion bypassed the requirement for Est3 (Fig. 1C) (*17*), showing that Est1 and Est3 perform functionally distinct roles in telomere replication.

Our results are consistent with a model in which Cdc13 mediates telomerase access by a direct interaction with the enzyme (Fig. 4). Furthermore, these data indicate that Est1 is a comediator of this "accessing" function, potentially as a direct binding partner of Cdc13, although we cannot rule out the possibility of

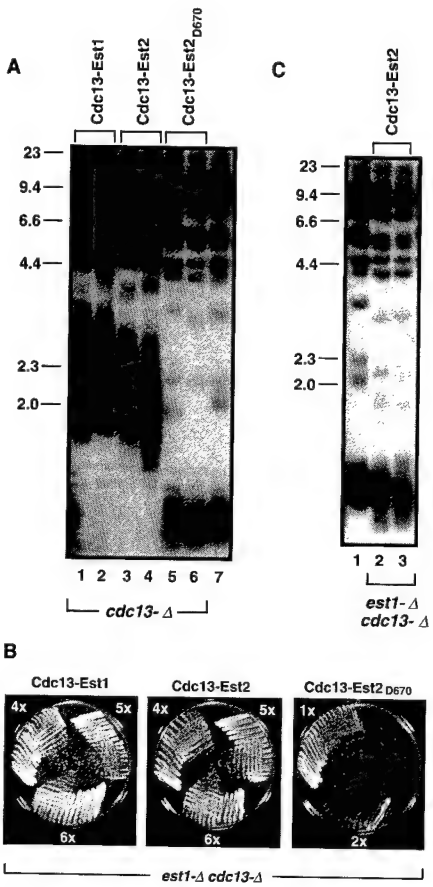

Fig. 3. Fusing Cdc13 to the catalytic subunit of telomerase bypasses the requirement for Est1. (**A**) Telomere length, after ~75 generations of growth. Lanes 1 and 2, *cdc13*-Δ/pVL1091; lanes 3 and 4, *cdc13*-Δ/pVL1107 (expressing the Cdc13-Est2 fusion); lanes 5 and 6, *cdc13*-Δ/pVL1111 (Cdc13-$Est2_{D670}$); lane 7, *CDC13*$^{+}$ control strain. (**B**) Growth of an *est1*−Δ *cdc13*-Δ strain with either pVL1091 or pVL1107 for ~100 (4×) to ~150 (6×) generations after eviction of the *CDC13* plasmid. The *est1*-Δ *cdc13*-Δ/pVL1111 (Cdc13-$Est2_{D670}$) strain (constructed by dissection of a *est1*-Δ/*EST1*$^{+}$ *cdc13*-Δ/*CDC13*$^{+}$ diploid strain with pVL1111) is shown after ~25 and ~50 generations of growth. (**C**) Telomere length, after ~150 generations of growth, of a *CDC13*$^{+}$ *EST*$^{+}$ control strain (lane 1) or an *est1*−Δ *cdc13*-Δ/pVL1107 strain (lanes 2 and 3). This strain has been propagated for an additional ~125 generations with no signs of senescence or changes in telomere length (*17*).

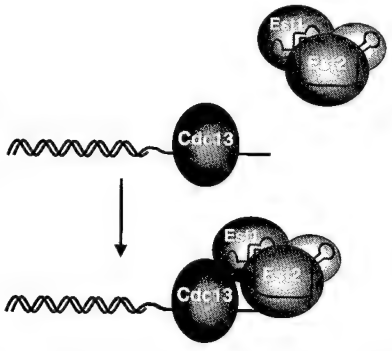

Fig. 4. Model for Cdc13 and Est1 as positive regulators of telomerase function. Cdc13 is proposed to bind the single-stranded overhang present at the ends of chromosomes and to mediate telomerase access by a direct but weak protein interaction with a component of the telomerase holoenzyme, possibly Est1. Telomerase is shown as a multisubunit RNA-containing complex that may include additional proteins such as Est3 (*14*). In addition to the positive regulation described here, telomeres are also subject to negative length regulation in both yeast and human cells, which has been proposed to be mediated by cis-inhibition of telomerase through the action of duplex telomere DNA binding proteins (*3*). Whether Est1 and Cdc13 are the direct recipients of such negative regulators is an intriguing question.

additional intervening protein or proteins. These experiments may have also uncovered an additional role for Est1 in telomerase function, as the Cdc13-Est2 fusion was not capable of promoting extensive telomere elongation in the absence of Est1 (Fig. 3C) (*26*). Because Est1 is a terminus-specific DNA binding protein (*11*), we speculate that this second role may be to promote accessibility of the 3′ terminus to the active site of telomerase.

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15. In-frame fusion proteins were constructed as follows: The Cdc13-Est1 fusion (pVL1091) fused $Cdc13_{1-924}$-$Est1_{6-699}$; the Cdc13-Est2 (pVL1107) and Cdc13-$Est2_{D670A}$ (pVL1111) proteins fused $Cdc13_{1-924}$-$Est2_{1-884}$. All three constructs were expressed from the genomic *CDC13* promoter and derived from the single-copy *CEN* plasmid, pRS415. The Est1-DBD_{Cdc13} and Est1-47-DBD_{Cdc13} fusions (pVL1120 and pVL1121, respectively), expressed in single copy from the *EST1* promoter, fused the Cdc13 DNA binding domain (*22*) to the COOH terminus of Est1, to generate $Est1_{1-699}$ - $Cdc13_{2-21,452-693}$. Telomere elongation by any of these fusion proteins is not due to increased protein expression in the context of the fusion, because overexpression of Est1, Est2, or Cdc13, or expression of Est1 by the *CDC13* promoter, has little or no effect on telomere length (*8*, *11*, *17*, *19*).
16. We have also observed up to a ~4-kb increase in the length of a single telomere (chromosome IIIL). Strains with greatly elongated telomeres do not exhibit any discernable growth defect but were not examined for more subtle defects (such as alterations in cell cycle progression). We have not investigated yet whether the increase in telomere length in strains carrying Cdc13-telomerase fusions occurs at a constant rate, or if there is some influence of cis-inhibition on elongation, as previously observed (*3*).
17. S. K. Evans and V. Lundblad, unpublished data.
18. The *est1-47* mutation is one of a panel of alanine-scan mutations in *EST1* (*17*); the Est1-47 mutant protein still retains association with telomerase (at 20% of WT levels), as assessed by coimmunoprecipitation with the TLC1 RNA (*27*).
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23. Previous work showed that high-level expression of the $Est2_{D670A}$ mutant protein (under the control of the *ADH* promoter, on a 2μ high-copy plasmid) resulted in substantially shorter telomeres in an *EST2*$^+$ strain (*8*). The lack of an effect of the Cdc13-$Est2_{D670A}$ fusion on WT telomere length (Fig. 3A) is presumably a consequence of the lower levels of this fusion protein (confirmed by protein immunoblotting analysis), due to single-copy plasmid expression by the *CDC13* promoter. As expected, the Cdc13-$Est2_{D670A}$ fusion failed to complement an *est2*-Δ strain.
24. Bypass of *est1*-Δ senescence was not simply a consequence of increased Est2 levels (due to possible minimal increase in expression of *EST2* by the *CDC13* promoter), because even higher level expression of *EST2* by the constitutive *ADH* promoter (*8*) was not sufficient to allow an *est1*-Δ strain to grow (*17*).
25. V. Lundblad and E. H. Blackburn, *Cell* **73**, 347 (1993).
26. Association of Cdc13-Est2 fusion protein with the TLC1 RNA was reduced by less than twofold in the absence of Est1, as assessed by immunoprecipitation (*27*), arguing that the failure to elongate telomeres in an *est1*-Δ strain is not simply due to reduced stability of the Cdc13-Est2 telomerase complex.
27. For each sample, cells were grown in selective media to an optical density (600 nm) of 1.0. Cells were harvested by centrifugation and the cell pellets were washed in water and then in TMG 300+ [10 mM tris-HCl (pH 8.0), 1 mM $MgCl_2$, 5% glycerol, 1 mM phenylmethylsulfonyl fluoride, 300 mM NaCl]. Cell extracts were prepared by five repeated cycles of freezing and grinding in liquid nitrogen. Extracts were cleared twice by centrifugation for 10 min at 14,000 rpm at 4°C and immunoprecipitated with an antibody to hemagglutinin (HA) (16β12, Babco) and protein A/G agarose beads (Calbiochem). RNA was prepared by SDS–phenol-chloroform extraction, and TLC1 was detected on 7 M urea–4% polyacrylamide gel as described (*8*). The efficiency of TLC1 recovery in immunoprecipitates is typically less than 2%; the recovery with untagged proteins was less than 0.05%.

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The Est3 protein is a subunit of yeast telomerase

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***EST1*, *EST2*, *EST3* and *TLC1* function in a single pathway for telomere replication in the yeast *Saccharomyces cerevisiae* [1,2], as would be expected if these genes all encode components of the same complex. Est2p, the reverse transcriptase protein subunit, and TLC1, the templating RNA, are subunits of the catalytic core of yeast telomerase [3–5]. In contrast, mutations in *EST1*, *EST3* or *CDC13* eliminate telomere replication *in vivo* [1,6–8] but are dispensable for *in vitro* telomerase catalytic activity [2,9]. Est1p and Cdc13p, as components of telomerase and telomeric chromatin, respectively, cooperate to recruit telomerase to the end of the chromosome [7,10]. However, Est3p has not yet been biochemically characterized and thus its specific role in telomere replication is unclear. We show here that Est3p is a stable component of the telomerase holoenzyme and furthermore, association of Est3p with the enzyme requires an intact catalytic core. As predicted for a telomerase subunit, fusion of Est3p to the high affinity Cdc13p telomeric DNA binding domain greatly increases access of telomerase to the telomere. Est1p is also tightly associated with telomerase; however, Est1p is capable of forming a stable TLC1-containing complex even in the absence of Est2p or Est3p. Yeast telomerase therefore contains a minimum of three Est proteins for which there is both *in vivo* and *in vitro* evidence for their role in telomere replication as subunits of the telomerase complex.**

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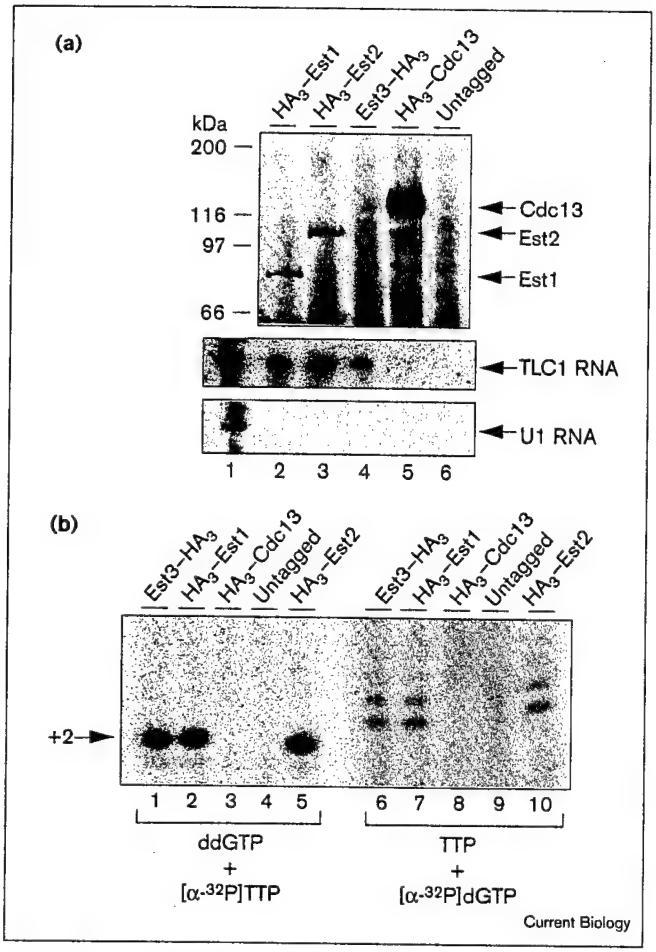
Results and discussion

To address whether Est3p is a component of telomerase, we examined whether it is associated with the TLC1 RNA and with enzyme activity, in parallel with Est1p, Est2p and Cdc13p. To avoid artifactual associations that might arise from over-expression, we constructed a set of strains containing epitope-tagged versions of each gene integrated into the respective endogenous locus under the control of the native promoter. The identical hemagglutinin $(HA)_3$ epitope was introduced into a region of each protein such that the activity of the tagged protein was not overtly affected; little or no alterations in telomere length were observed in strains bearing integrated versions of HA_3–*EST1*, HA_3–*EST2*, *EST3*–HA_3 or HA_3–*CDC13* (see Supplementary material).

Immunoprecipitates (IPs) from extracts prepared from these four individually tagged strains were assayed for the presence of the relevant recombinant protein, the TLC1 RNA subunit and telomerase activity (Figure 1). HA_3–Est1, HA_3–Est2, and HA_3–Cdc13 proteins were detected in the IPs (Figure 1a), but were not visible in the starting extracts (data not shown), presumably due to the low levels of each of these proteins when expressed under their native promoter. The TLC1 RNA was readily detectable in Est1p, Est2p and Est3p IPs by northern analysis, but was not present in IPs prepared from an untagged strain (Figure 1a). The interactions with TLC1 were specific for the telomerase RNP, since the U1 small nuclear RNP (snRNP) RNA was not present in each IP (Figure 1a).

This interaction between the Est proteins and TLC1 reflected an association with an active telomerase complex, since enzyme activity was identified in each of the Est protein IPs (Figure 1b). Two related assays, which have previously been used to monitor telomerase from *S. cerevisiae* [2,4,9], were used to assess enzyme activity. In the first assay, primer extension by telomerase was conducted in the presence of the chain-terminating ddGTP nucleotide, thereby concentrating the signal into a +2 product and providing a sensitive means of detecting enzyme activity [2,4]. Telomerase activity was readily observed in each Est protein IP using this assay (Figure 1b, lanes 1–5). In several repetitions of this experiment, the levels of enzyme activity correlated directly with the levels of TLC1 and varied by no more than two-fold when compared among the three Est protein IPs (data not shown), as predicted if these three proteins, each containing the identical HA_3 epitope, are subunits of the same complex. Enzyme activity was also monitored using a non-terminating reaction (Figure 1b, lanes 6–10) that generates a ladder of reaction products representing the

Figure 1



Est1, Est2 and Est3 proteins associate with an active telomerase enzyme complex. **(a)** Extracts from an isogenic set of strains, each bearing the same HA_3 epitope tag on a single gene as indicated, were immunoprecipitated and analyzed by western blotting to detect HA tagged protein (top) and northern blotting to detect TLC1 RNA (middle) or U1 RNA (bottom). HA_3–Est1 (strain TVL288; lane 2); HA_3–Est2 (TVL292; lane 3); Est3–HA_3–GST (TVL293; lane 4); HA_3–Cdc13 (TVL290; lane 5); untagged (AVL78; lane 6). TLC1 and U1 RNA levels in crude extracts prepared from an untagged strain, prior to immunoprecipitation, are shown in lane 1; the amount of extract represents 4% of the amount loaded in the IP lanes. Lanes 2–6 are equivalently loaded, as assessed by total protein. The Est3–HA_3–GST polypeptide co-migrates with the antibody heavy chain (which is not shown) and is not detectable on this particular blot. **(b)** Immunoprecipitates from each tagged strain were assayed for telomerase activity with the primer substrate (5′-TGTGGTGTGTGT-GGG-3′), using either a chain terminating reaction in the presence of ddGTP (lanes 1–5; the band representing the +2 product is indicated) or non-chain terminating reaction (lanes 6–10). The strains used in this experiment are identical to those used in (a), with the exception of the Est3–HA_3 strain (TVL307). Telomerase activity was RNase A-sensitive and primer-dependent in both sets of assays (data not shown).

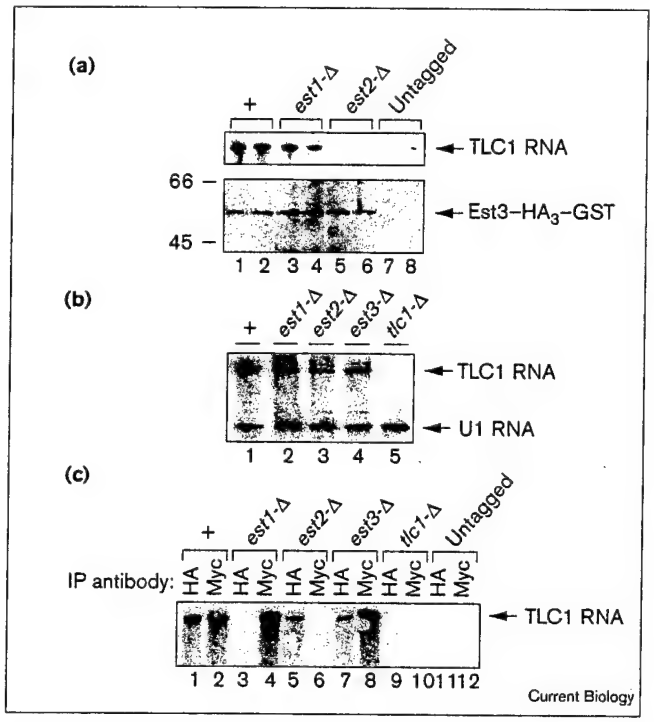
incomplete extension of a primer across the TLC1 template [9,11]. Telomerase activity was again detected in HA_3–Est1p and Est3p–HA_3 IPs at roughly the same levels as observed in HA_3–Est2p IPs (compare lanes 6 and 7 with lane 10). In both protocols, IPs were subjected to relatively stringent wash conditions (0.4 M NaCl) prior to enzyme assays. Therefore, Est3p is tightly associated with an active telomerase complex, indicating that Est3p plays its role in telomere replication as a subunit of telomerase. These experiments also show that Est1p is a stable component of the enzyme, confirming and extending previous observations which showed that Est1, when overexpressed, was associated with the TLC1 RNA [12–14].

In contrast, neither the TLC1 RNA subunit (Figure 1a) nor enzyme activity (Figure 1b) were detectable in Cdc13p IPs performed under the same high stringency conditions. Even under less stringent conditions, telomerase activity was not detected in Cdc13p IPs (data not shown). Thus, Cdc13p is not tightly associated with telomerase, although these observations do not exclude the possibility of a transient interaction.

To determine the requirements for interaction of Est3p with telomerase, we examined whether Est3p association with a TLC1-containing complex could be retained in the absence of either Est1p or Est2p. Deletion of *EST1* or *EST2* did not affect the level of Est3–HA_3–GST protein recovered from whole cell extracts on glutathione beads (Figure 2a). In addition, the steady state levels of TLC1 RNA, which were normalized to the levels of U1 RNA, were also unchanged in extracts prepared from strains that lacked either Est1p, Est2p or Est3p (Figure 2b). When immunoprecipitated, Est3p retained association with a TLC1-containing complex in a strain deleted for *EST1* (Figure 2a). However, the ability of Est3p to associate with TLC1 was abolished in the absence of the Est2p subunit (Figure 2a), indicating that an intact catalytic core is a requirement for the interaction of Est3p with the telomerase complex. Whether this reflects a direct interaction between the Est2 and Est3 proteins has not yet been determined; two hybrid tests intended to detect such an interaction have been negative so far (D.K. Morris and V.L., unpublished observation).

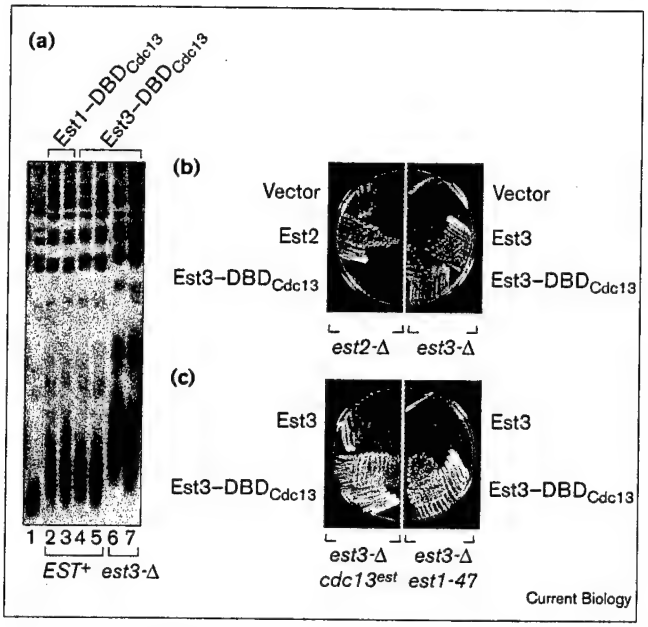
We similarly investigated the requirement for the association of Est1p with telomerase. For these experiments, we constructed a strain containing differentially tagged versions of Est1p and Est2p (HA_3–Est1p and Myc_3–Est2p) integrated into the genome and expressed under the native *EST1* or *EST2* promoters. Est3p was not required for the interaction of Est1p with the enzyme (Figure 2c), indicating that Est1p and Est3p associate with telomerase independently of each other. Unlike Est3p, however, the Est1 protein was capable of forming a TLC1-containing complex even in the absence of the catalytic Est2p subunit (Figure 2c), indicating that Est1p and Est2p interact with TLC1 independently of one another. Similar results for Est2p- and Est3p-independent association of

Figure 2



The association of Est3p with the telomerase RNP requires an intact catalytic core. **(a)** Top: extracts prepared from an Est3–HA_3–GST strain (TVL293) or deletion derivatives of TVL293 were immunoprecipitated, and TLC1 RNA was detected by northern blotting. TVL293 (lanes 1,2); *est1-Δ* derivative of TVL293 (lanes 3,4); *est2-Δ* derivative of TVL293 (lanes 5,6); untagged (AVL78; lanes 7,8). Bottom: extracts were purified on glutathione sepharose and analyzed by western blotting with an antibody to detect the HA_3-tagged Est3 protein; the GST-pulldown assay was used to determine levels of the Est3 protein, since Est3p comigrated with the immunoglobulin heavy chain when immunoprecipitated. **(b)** Extracts prepared from *est1-Δ* (lane 2), *est2-Δ* (lane 3), *est3-Δ* (lane 4) and *tlc1-Δ* strains (lane 5), in parallel with an *EST*$^+$ strain (lane 1), were analyzed by northern blotting to detect TLC1 RNA and U1 RNA, followed by quantification of the relative RNA levels by PhosphorImager analysis. The abundance of TLC1 RNA was reduced by no more than three-fold in the three *EST* deletion strains, relative to the ratio of the two RNAs in a wild-type strain. **(c)** Extracts from TVL300 (containing HA_3–Est1p and Myc_3–Est2p) or deletion derivatives of strain TVL300 were immunoprecipitated with either anti-Myc or anti-HA antibodies and analyzed by northern blotting to detect *TLC1* RNA. TVL300 (lanes 1,2); *est1-Δ* derivative of TVL300 (lanes 3,4); *est2-Δ* derivative of TVL300 (lanes 5,6); *est3-Δ* derivative of TVL300 (lanes 7,8); *tlc1-Δ* derivative of TVL300 (lanes 9,10); untagged (AVL78; lanes 11,12). The variations in TLC1 signal in HA versus Myc immunoprecipitations are due, at least in part, to the fact that the Myc_3 tag is a more efficiently immunoprecipitated epitope, as Myc_3–Est2p co-immunoprecipitates more telomerase RNA than HA_3–Est2p (data not shown).

Figure 3



The Est3–DBD_{Cdc13} fusion increases access of telomerase to the chromosome terminus. **(a)** Genomic Southern blot hybridized with a telomere-specific probe, as described previously [1]; DNA was prepared after ~50 generations of growth following plasmid transformation. *EST*$^+$ control (lane 1); *EST*$^+$/pVL1120 (Est1–DBD_{Cdc13}; lanes 2,3); *EST*$^+$/pVL1292 (Est3–DBD_{Cdc13}; lanes 4,5); *est3-Δ*/pVL1292 (Est3–DBD_{Cdc13}; lanes 6,7); all plasmids were single copy CEN vectors and expressed under the native *EST* gene promoter. **(b)** Growth after approximately 50 generations of *est2-Δ* or *est3-Δ* strains harboring either vector or single copy plasmids expressing the appropriate wild-type Est protein or the Est3–DBD_{Cdc13} fusion. **(c)** Growth after ~50 generations of an *est3-Δ cdc13-2*^{est} strain or an *est3-Δ est1-47* strain with plasmids expressing the wild-type Est3 protein or the Est3–DBD_{Cdc13} fusion. These strains expressing the Est3–DBD_{Cdc13} fusion have been propagated a further 75 generations with no signs of senescence.

Est1p with a TLC1 complex have been reported, using an over-expressed LexA–Est1 protein fusion [14].

We have previously shown that telomerase access to the telomere can be greatly augmented by fusing the high affinity DNA binding domain of Cdc13p (DBD_{Cdc13}) to Est1p, which results in substantial telomere lengthening [10]. The demonstration that Est3p, like Est1p, is also telomerase-associated predicts that fusion of the DBD_{Cdc13} to Est3p should similarly enhance telomerase access and consequently increase telomere length. Indeed, an Est3–DBD_{Cdc13} fusion, expressed under the *EST3* promoter and on a single copy plasmid, elongated telomeres in a wild-type strain, comparable to the lengthening conferred by the Est1–DBD_{Cdc13} fusion (Figure 3a). This fusion protein complemented a strain in which *EST3* had been deleted (*est3-Δ*; Figure 3b), demonstrating that the Est3 portion of the fusion was still functional. Telomere elongation was further enhanced in an *est3-Δ* strain (Figure 3a), presumably due to the absence of the competing wild-type Est3 protein. The effect on telomere length was telomerase-dependent, as the Est3–DBD_{Cdc13} fusion failed to bypass the telomere replication defect of an *est2-Δ* strain (Figure 3b). Therefore, telomerase can be delivered to the

chromosome end by fusing either of two telomerase-associated proteins, Est1p or Est3p, to the DBD_{Cdc13}. This is a specific consequence of joining a telomerase subunit to the DBD_{Cdc13}, since expression of either the DBD_{Cdc13} alone or as a fusion to two other proteins implicated in telomere length maintenance (Stn1 and Pol1) [15,16] does not result in telomere elongation (S.K.E., A. Chandra, E. Pennock and V.L., unpublished observations).

We further tested the ability of the Est3–DBD_{Cdc13} fusion to confer enzyme access in the absence of the telomerase-mediating functions of Cdc13p and Est1p. Two previously described mutations, *cdc13-2^{est}* and *est1-47*, appear to specifically compromise the ability of telomerase to access the end of the chromosome [10]. This is based in part on previous observations showing that first, the *cdc13-2^{est}* mutant phenotype can be bypassed by the Est1–DBD_{Cdc13} fusion, and second, the telomere replication defect of the Est1-47 protein can be bypassed by fusion of Est1-47p to the DBD_{Cdc13} [10]. The Est3p–DBD_{Cdc13} fusion was similarly effective in bypassing the senescence phenotype of both the *cdc13-2^{est}* and *est1-47* mutations (Figure 3c). This indicates that fusing the DBD_{Cdc13} to a telomerase-associated protein can alleviate the requirement for the telomerase accessing functions of either Cdc13 or Est1. However, the Est3–DBD_{Cdc13} fusion was not capable of rescuing the telomere replication defect of an *est1-Δ* null strain, and an Est1–DBD_{Cdc13} fusion was similarly unable to rescue an *est3-Δ* strain (data not shown). Thus, neither fusion is sufficient to bypass the complete function of the other protein.

The demonstration that the Est3 protein is a component of telomerase completes the prediction for the functions of *EST1*, *EST2*, *EST3* and *TLC1* based on PPgenetic observations. Previous epistasis analysis had shown that these four genes function in a single pathway for telomere replication, as would be expected if these genes each encode subunits of the same enzyme complex [1,2]. The work presented here, combined with previous observations, shows that all four genes encode components of telomerase. A role for the Est1 protein subunit in telomerase recruitment has been previously proposed, acting as a bridging protein between telomerase and the telomere. Additional genetic and biochemical investigation should help reveal the precise biochemical activity of the Est3 telomerase protein subunit.

Supplementary material

Supplementary material including details of strain construction and additional methodological procedures is available at http://current-biology.com/supmat/supmatin.htm.

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COMMENTARY

Positive and negative regulation of telomerase access to the telomere

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SUMMARY

The protective caps on chromosome ends – known as telomeres – consist of DNA and associated proteins that are essential for chromosome integrity. A fundamental part of ensuring proper telomere function is maintaining adequate length of the telomeric DNA tract. Telomeric repeat sequences are synthesized by the telomerase reverse transcriptase, and, as such, telomerase is a central player in the maintenance of steady-state telomere length. Evidence from both yeast and mammals suggests that telomere-associated proteins positively or negatively control access of telomerase to the chromosome terminus. In yeast, positive regulation of telomerase access appears to be achieved through recruitment of the enzyme by the end-binding protein Cdc13p. In contrast, duplex-DNA-binding proteins assembled along the telomeric tract exert a feedback system that negatively modulates telomere length by limiting the action of telomerase. In mammalian cells, and perhaps also in yeast, binding of these proteins probably promotes a higher-order structure that renders the telomere inaccessible to the telomerase enzyme.

Key words: Telomere, Telomerase access, Positive length regulation, Negative length regulation, Cdc13, Lagging strand synthesis, Rap1, TRF1, TRF2

INTRODUCTION

The ends of eukaryotic chromosomes are capped by protective structures known as telomeres. In most species, these termini are composed of arrays of short G-rich DNA repeats that are complexed with associated proteins (reviewed by Greider, 1991). Telomeres are essential for maintaining genomic stability: loss of normal telomere function can lead to end-to-end fusions and chromosome loss (Ahmed and Hodgkin, 2000; Blasco et al., 1997; Sandell and Zakian, 1993; van Steensel et al., 1998). Shortening of the telomeric tract below a certain critical length also limits long-term cellular proliferation; consequently, telomere shortening has implications for oncogenesis and potentially for cellular aging in multicellular organisms (reviewed by Harley and Villeponteau, 1995). The cell therefore employs mechanisms to ensure proper telomere function, including stable maintenance of the length of the telomeric tract. A complex set of mechanisms sense and regulate telomere length; here, we discuss several levels at which this regulation can occur.

In several organisms, such as the ciliated protozoa, the length of the telomeric repeat tract is precisely defined, but, in most species, telomere length is maintained within a particular size range as a result of a dynamic equilibrium between lengthening and shortening activities (reviewed by Greider, 1996). Telomere shortening can occur because of either incomplete replication of the ends or telomere-specific processing activities such as active degradation or deletion events. Sequence loss is counterbalanced by the addition of telomeric sequences by the enzyme telomerase, which synthesizes telomeric repeats onto the G-rich strand. Despite these opposing lengthening and shortening activities, telomeres can be maintained in certain cell types such that there is no net sequence gain or loss. For example, a telomerase-proficient yeast strain propagated for thousands of cell divisions shows no gross variation in telomere length (Shampay and Blackburn, 1988). However, altering the levels of either telomerase or telomere-shortening activities, or preventing/promoting the ability of these activities to access the telomere, can modulate this balance, which results in changes in the steady-state telomere length.

Telomerase is a central player in the maintenance of steady-state telomere length and is therefore a principal target for both positive and negative regulatory mechanisms. Although telomerase function can be modulated in a variety of ways (for example, by regulating the levels, assembly or activity of the enzyme), we focus here primarily on the regulation of access of telomerase to the chromosome terminus. Other mechanisms can maintain chromosome termini in the absence of telomerase (Bryan et al., 1995; Cohn and Edstrom, 1992; Lundblad and Blackburn, 1993; McEachern and Blackburn, 1996; Nakamura et al., 1998; Sheen and Levis, 1994; Teng and Zakian, 1999). In particular, increasing attention has been directed at the potential parallels between a recombination-based pathway for

telomere maintenance described in yeast and the ALT pathway in human cells (see Kass-Eisler and Greider, 2000, for information on this subject).

POSITIVE REGULATION OF TELOMERE LENGTH: RECRUITMENT OF TELOMERASE TO THE TELOMERE

Telomerase is a reverse transcriptase that synthesizes telomeric repeats by using an internal RNA subunit as the template to dictate the sequence added to chromosome ends (reviewed by Nugent and Lundblad, 1998). In budding yeast, *TLC1* and *EST2* encode the template RNA and catalytic reverse transcriptase subunits, respectively, of the catalytic core of the telomerase enzyme (Counter et al., 1997; Lingner et al., 1997b; Singer and Gottschling, 1994). Strains lacking either of these two genes exhibit a telomere replication defect that is a hallmark of a telomerase deficiency: progressive shortening of telomeres and an accompanying gradual decline in cell viability, referred to as the Est$^-$ phenotype (for Ever Shorter Telomeres; Lundblad and Szostak, 1989). As expected by analogy to other polymerases, evidence from several different systems indicates that telomerase exists as a holoenzyme complex, and that associated subunits potentially confer either additional in vivo properties or augmented activity (e.g. the ability to respond appropriately to different substrates). In yeast, mutations in three additional genes – *EST1*, *EST3* and *CDC13* – confer the same Est$^-$ phenotype (Lendvay et al., 1996; Lundblad and Szostak, 1989; Nugent et al., 1996), although extracts prepared from these three strains have telomerase catalytic activity (Cohn and Blackburn, 1995; Lingner et al., 1997a). The apparent discrepancy between the in vivo and in vitro requirements for these three proteins defines Est1p, Est3p and Cdc13p as positive regulators of telomerase. Biochemical analysis has shown that Est1p and Est3p execute their role(s) in telomere replication as subunits of the telomerase holoenzyme, whereas Cdc13p, which is not tightly associated with telomerase, is a component of telomeric chromatin (Bourns et al., 1998; Evans and Lundblad, 1999; Hughes et al., 2000; Lin and Zakian, 1995; Nugent et al., 1996; Steiner et al., 1996).

Although the specific function of the Est3p telomerase subunit has not yet been determined, a series of studies have shown that Est1p and Cdc13p, as components of telomerase and telomeric chromatin, respectively, function as co-mediators of access of telomerase to chromosome termini. Both proteins bind single-strand telomeric substrates, which is consistent with a role for each at the telomere (Lin and Zakian, 1996; Nugent et al., 1996; Virta-Pearlman et al., 1996). However, unlike Cdc13p, Est1p requires a free 3′ terminus in order to bind to telomeric substrates, which suggests that a primary function of this telomerase subunit is to mediate interaction between the enzyme and the end of the chromosome (Virta-Pearlman et al., 1996). Telomere replication also requires the participation of Cdc13p, as revealed by the identification of a specific missense mutation, *cdc13-2^{est}*, which has a telomere replication defect even though telomerase catalytic activity is unaffected (Lingner et al., 1997a; Nugent et al., 1996). These observations have led to the proposal that Cdc13p recruits telomerase to the telomere through a direct (albeit weak) association with the enzyme and that this activity is abolished by the *cdc13-2^{est}* mutation. This defect can be bypassed if the high-affinity DNA-binding domain of Cdc13p (DBD_{Cdc13}) is fused directly to either Est1p or Est3p (Evans and Lundblad, 1999). In a *cdc13-2^{est}* strain carrying either DBD_{Cdc13}-telomerase fusion, telomere replication is restored, which suggests that a DBD_{Cdc13}-telomerase fusion does not require the recruitment function of Cdc13p to access the telomere. An obvious candidate for the telomerase component that participates in this recruitment step is Est1p. Overexpression of the wild-type Est1p protein partially suppresses the telomere replication defect of the *cdc13-2^{est}* mutant (Nugent et al., 1996), and Est1p and Cdc13p can be co-immunoprecipitated when both are overexpressed in yeast (Qi and Zakian, 2000), which indicates that increased Est1p levels can enhance the interaction between the two proteins. Furthermore, a fusion between Cdc13p and the catalytic core of telomerase allows telomeres to be stably maintained in the absence of Est1p, which is consistent with a role for Est1p as a bridging molecule that mediates access of the enzyme to the chromosome terminus (Evans and Lundblad, 1999). Collectively, these experiments implicate Est1p and Cdc13p as collaborators in positive regulation of access of telomerase to the telomere (Fig. 1).

Studies of telomerases from *Euplotes*, *Tetrahymena* and mammalian systems have uncovered other potential telomerase components, on the basis of either biochemical association with the ribonucleoprotein particle (RNP) or molecular assays designed to detect additional subunits (Collins et al., 1995; Harrington et al., 1997; Le et al., 2000; Lingner and Cech, 1996; Mitchell et al., 1999b; Nakayama et al., 1997; Seto et al., 1999). Features of several of these proteins suggest that their contribution to telomerase function may be in ribonucleoprotein structure or assembly (Kickhoefer et al., 1999; Le et al., 2000; Mitchell et al., 1999a; Mitchell et al., 1999b; Seto et al., 1999). None of these proteins shows sequence similarity to the yeast holoenzyme subunits described above, and no homologs of Est1p, Est3p or Cdc13p proteins have been recovered by other means. However, this may be due to incomplete databases or the current limits of search tools designed to identify homologs. Therefore, there is insufficient information to indicate whether telomerase access at human telomeres will be subject to a positive regulatory mechanism similar to that described for budding yeast. However, a comparison between a particular class of mutation isolated in the catalytic subunits of both human and yeast telomerases provides a potential hint. Friedman and Cech have identified a unique 'separation of function' mutation in the reverse transcriptase subunit of yeast telomerase that is defective for telomere replication but retains enzyme activity, which suggests that the catalytic subunit is disrupted for its interaction with a holoenzyme component (Friedman and Cech, 1999). A phenotypically similar defect has also been identified in hTERT, the human reverse transcriptase telomerase protein: when this allele of *hTERT* is transfected into telomerase-deficient cells, telomerase enzyme activity is restored but telomeres are not maintained (Counter et al., 1998; Ouellette et al., 1999). This suggests that there are factors in human cells that will similarly regulate access of telomerase to the telomere.

An additional important aspect of telomere replication is the

generation of the G-rich single-strand extension that appears to be a ubiquitous feature of telomeres (McElligott and Wellinger, 1997; Wellinger et al., 1993). In yeast, the regulation of the production of this structure has two related implications for telomere replication. First, telomerase is not capable of extending a blunt-ended DNA molecule in vitro (Lingner and Cech, 1996). Therefore, following conventional DNA replication, the blunt-ended chromosome end that is created as a consequence of leading strand synthesis must be processed to create a single-strand extension prior to telomerase action. Since this processing reaction should also provide a substrate for Cdc13p binding, the generation of a single-strand G-tail is likely to be an important step for recruitment of the telomerase enzyme as well as for efficient telomere addition. Generation of the G-tail is still a complex and poorly understood aspect of telomere replication, and the enzymatic activity(s) (such as a postulated exonuclease) responsible for creating such a structure is still unknown.

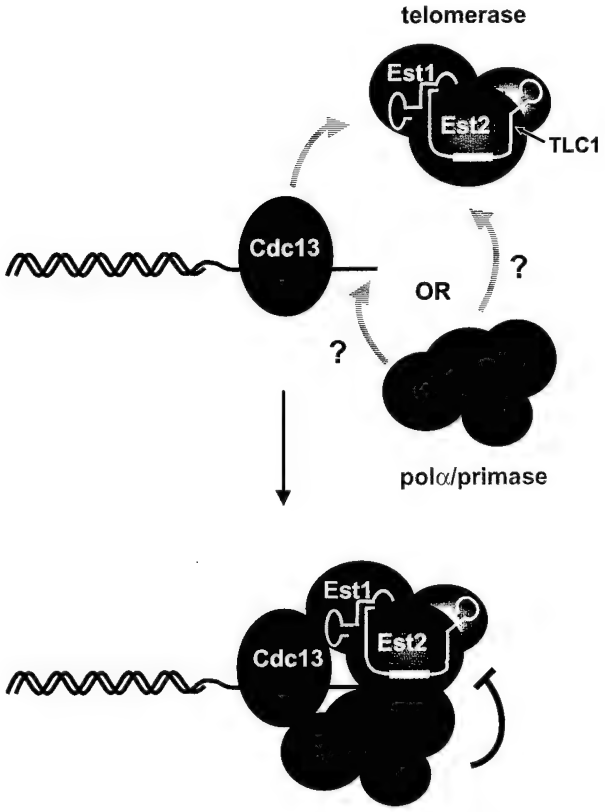

Fig. 1. Positive regulation of telomere length requires recruitment of two polymerase complexes to the telomere. Telomerase recruitment is thought to be mediated by a direct protein-protein interaction between Cdc13p and the telomerase-associated Est1p protein. Est1p can form a complex containing TLC1 (the telomerase RNA) in the absence of other components of the holoenzyme (Hughes et al., 2000; Zhou et al., 2000), which suggests that there are separate determinants for interaction between TLC1 and different subunits of the telomerase RNP. The secondary structure for TLC1 RNA has not been determined; therefore, the indicated structure is hypothetical, although there may be a conserved stem-loop structure 5′ of the template region – by analogy with the closely related budding yeast *K. lactis* RNA (Tzfati et al., 2000). The 3′ chromosome terminus is shown bound to the active site, but Est1p has a ssDNA-binding activity that might contribute to the interaction of telomerase with the primer substrate (Virta-Pearlman et al., 1996). Details of the proposed recruitment of the lagging strand synthesis machinery are not known; here, recruitment of the polα-primase complex is shown to be coincident with recruitment of telomerase. It is also not known which proteins participate in this recruitment; a direct interaction between a component of the telomerase RNP or Cdc13p (and/or its associated proteins) might function in this capacity (see text for details). Although recruitment of lagging strand synthesis machinery is a putative positive regulatory step required for telomerase function, further elongation by telomerase might actually be inhibited by C strand synthesis, as indicated.

SYNTHESIS OF THE OTHER STRAND OF THE TELOMERE: POSITIVE AND NEGATIVE REGULATION CONVERGE

Although the initial focus has been on telomerase as a principal target for telomere length regulation, several recent studies indicate that coordination between C strand and G strand synthesis is required for proper telomere length maintenance. During de novo telomere synthesis in the ciliate *Euplotes*, addition of aphidicolin, which inhibits DNA polymerases α and δ but not telomerase, not only leads to changes in the length of the C strand but also causes an increase in both the length and heterogeneity of the G strand (Fan and Price, 1997). Similarly, growth of an *S. cerevisiae* strain carrying conditional lethal mutations in DNA polymerase α at the semi-permissive temperature results in a telomerase-dependent increase in telomere length, and such strains exhibit an increase in the length of the G strand relative to the C strand (Adams Martin et al., 2000; Carson and Hartwell, 1985). This suggests that synthesis of the C strand is normally coordinated with elongation of the G strand, but partial impairment of C strand synthesis allows telomerase to extend the G strand beyond what is normally observed. This coordination again may require the participation of Cdc13p and the Cdc13p-associated protein Stn1p. Mutations in either *CDC13* or *STN1* have been isolated that result in telomerase-dependent elongation of the G strand of the telomere, which is similar to the consequences of defects in DNA polymerase α (Grandin et al., 1997; Qi and Zakian, 2000; A. Chandra, T. R. Hughes and V. Lundblad, unpublished data). Furthermore, Cdc13p is physically associated with DNA polymerase α (Qi and Zakian, 2000). Therefore, Cdc13p both negatively and positively regulates telomere length in yeast. One model to explain these dual regulatory roles is that Cdc13p first recruits telomerase to the telomere and subsequently acts to limit the extension of the G strand by telomerase in response to synthesis of the C strand by the DNA-polymerase-α–primase complex.

Two additional assays, which monitor the behavior of specific chromosome termini, have also implicated C strand synthesis in telomere replication. In the first assay, which measures de novo telomere formation, cleavage by a double-strand endonuclease at a site adjacent to a short telomeric tract is followed by efficient addition of telomeric DNA onto the newly exposed double-strand break (Diede and Gottschling, 1999). Telomere formation can be monitored at a molecular level, if cells are arrested in the G_2/M stage of the cell cycle by the drug nocodozole (although, as noted below, telomerase elongation of chromosome termini does not occur during this

phase of the cell cycle). As expected, telomere addition requires *EST1-3*, *TLC1* and the telomerase recruitment function of *CDC13*. Strikingly, elimination of DNA polymerases α and δ or DNA primase (through the use of thermolabile mutations) also abolishes elongation of this terminus, and there is no detectable synthesis of the G strand. Diede and Gottschling therefore have proposed that the telomerase holoenzyme must interact with one or more components of the lagging strand synthesis machinery in order to elongate chromosomal termini, and that G strand synthesis must consequently be tightly coupled to synthesis of the C strand (Fig. 1). This model predicts that recruitment of the machinery responsible for replication of the C strand might be as important for telomere maintenance as recruitment of telomerase, and that defects in this proposed second recruitment step should also give rise to an Est$^-$ phenotype – by analogy with the proposed telomerase-recruitment defect of the *cdc13-2^{est}* mutant. Thus, either Cdc13p (Qi and Zakian, 2000) or even a telomerase component might have a role in recruiting lagging strand synthesis machinery to the telomere or coupling of synthesis by the two polymerase machines.

At first glance, these studies have produced apparently contradictory observations regarding the consequences of lagging strand replication defects: a complete defect in a component of the lagging strand machinery prevents G strand synthesis, whereas a partial impairment results in extended G strand synthesis. These two observations could be explained if synthesis of the C strand provides an opportunity for both positive and negative regulation of G strand synthesis by telomerase, at two successive steps. For example, as proposed in Fig. 1, recruitment of the lagging strand synthesis machinery could be essential for telomerase enzymatic activity – perhaps if components of the lagging strand machinery are absent, the whole complex is not formed, and telomerase cannot elongate the telomere. The extent of synthesis of the G strand might subsequently be negatively regulated by C strand synthesis, such that initiation of C strand synthesis acts as a signal to halt G strand synthesis. If synthesis of this second strand is delayed or reduced, this might remove limits on the extent of G strand DNA that is synthesized by telomerase.

A different assay, however, has been used to follow the elongation of a natural, but experimentally shortened, chromosome terminus (Marcand et al., 1999; Marcand et al., 2000); the results are somewhat different from those obtained in the de novo healing assay. In this system, shortening is induced by an internal recombination event within the G-rich telomeric repeat sequences, thereby leaving the end structure (and terminus-specific regulation) intact. When this terminus is monitored through the cell cycle, telomere elongation by telomerase occurs only in late S phase and coincides with the timing of telomeric DNA replication. No elongation is observed in nocodazole-blocked cells, in contrast to the results described above for de novo telomere formation. However, if the shortened telomere is present on a replication-defective plasmid, elongation is substantially reduced, which indicates that DNA replication through the telomere (including, presumably, lagging strand synthesis) is required for normal telomerase action. This requirement for DNA replication may reflect, at least in part, the need to generate a substrate for Cdc13p binding and/or telomerase synthesis, since appearance of the extended G-tails observed late in S phase requires passage of a replication fork (Dionne and Wellinger, 1998). Therefore, both this system and the de novo telomere addition assay indicate that telomere elongation is coupled to C strand synthesis. However, it is important to note that the telomere healing assay does not rely on passage through S phase; thus this assay, which incorporates features of double strand break repair, may influence the degree to which telomere synthesis relies on the lagging strand machinery.

DUPLEX TELOMERIC DNA-BINDING PROTEINS COUNT: NEGATIVE REGULATION OF TELOMERE LENGTH

The results described above indicate that, at least in budding yeast, a mechanism for recruiting telomerase to the chromosome terminus is necessary for telomere replication. However, in cells in which this recruitment mechanism is functional, what prevents telomeres from continuously elongating rather than maintaining the observed steady-state length? Results from studies in yeast and human cells point to an additional level of negative regulation, whereby the telomere itself modulates elongation by telomerase when telomere length exceeds a certain threshold. The current model proposes that duplex telomere DNA-binding proteins are part of a length-sensing mechanism that can discriminate the number of duplex-binding proteins bound to the telomere. Protein counting thereby provides the basis for a negative feedback system that modulates telomere length by inhibiting access of the telomerase enzyme through sequestration of the chromosome terminus. Because both yeast and mammalian duplex-binding proteins bend DNA, one model proposes a number-dependent switch that alters the equilibrium at the telomere between a telomerase-accessible 'open' structure and a 'closed' conformation that telomerase cannot access effectively (Fig. 2). The result is an exquisitely balanced cis-acting mechanism that finely tunes the length of individual telomeres. How this length-sensing mechanism influences activities that occur at the chromosome terminus – which lengthen the telomere tract accordingly – is not well understood. The proposed gradual folding into a restrictive higher-order conformation might limit the probability that telomerase can act on its substrate or might directly influence the enzyme by limiting the number of bases that telomerase can add in one addition cycle. One possibility is that Cdc13p or a subunit of telomerase is a direct target of this negative regulation.

In budding yeast, the major effector that negatively regulates telomere length is Rap1p, a duplex telomeric-DNA-binding protein that binds with high affinity to tandem GGTGT sites through two Myb-like domains (Buchman et al., 1988; Konig et al., 1996). Rap1p binding contributes to the formation of a complex called the telosome; assembly of this complex is mediated in part through the C-terminal domain of Rap1p, which interacts with two proteins, Rif1p and Rif2p, that contribute to telomere length control (Hardy et al., 1992; Wotton and Shore, 1997). The counting model described above stems from experiments in which targeting additional copies of the Rap1p C terminus to an individual telomere results in shortening of the terminal $G_{1\text{-}3}T$ tract at that telomere (Marcand et al., 1997; Ray and Runge, 1999). The extent of length reduction is roughly proportional to the number of targeted molecules, which indicates that the total number of

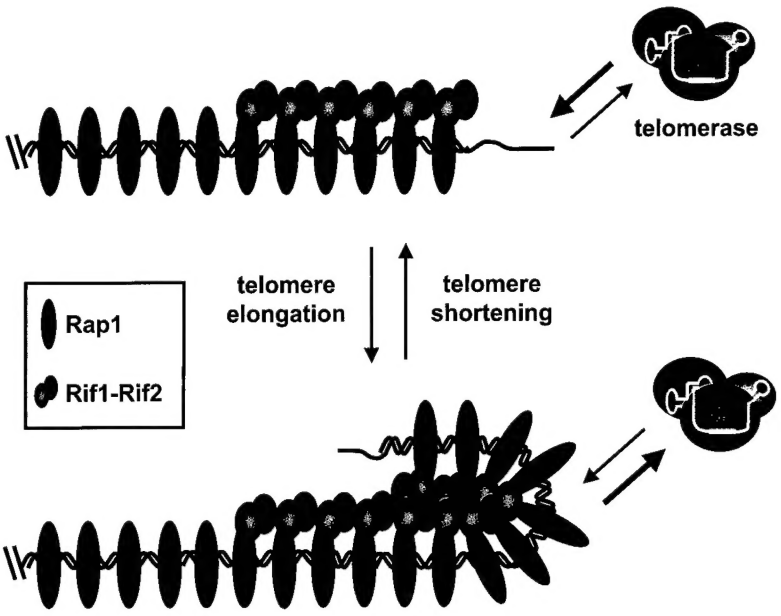

Fig. 2. Rap1p modulates telomere length by inhibiting access of the telomerase enzyme through sequestration of the chromosome terminus. Yeast telomeres contain high-affinity Rap1p-binding sites at a periodicity of approx. 18 bp (Gilson et al., 1993). When telomere length exceeds a certain threshold, the binding of excess Rap1p molecules is thought to shift the equilibrium at the telomere into a 'closed' conformation that is refractory to telomere elongation. Since Rap1p bends DNA, one proposed model for the 'closed' telomere conformation is a folded-back structure that renders the 3′ end inaccessible to telomerase. The action of Rap1p in effecting regulation is mediated through two proteins, Rif1p and Rif2p, which interact with the C terminus of Rap1p and also with each other (Wotton and Shore, 1997); therefore the folded structure might be stabilized through Rif-Rif protein interactions. When the telomere shortens below a critical level, presumably owing to incomplete telomere replication, one or more Rap1p molecules are lost from the telomere, which then shifts the equilibrium at the terminus towards an 'open' structure permissive to telomere addition. Whether this end structure forms a large duplex loop, as observed for mammalian telomeres (see Fig. 3), remains to be determined. Whether Cdc13p is constitutively present at chromosome termini, or whether the 'closed' conformation structure removes Cdc13p from the end, is also not known. Rap1 also associates with a complex of proteins required for transcriptional repression (the Sir proteins), which are not depicted in the figure and are not discussed in this review (for information on this subject, see Grunstein, 1998).

Rap1p molecules bound to a terminus, rather than G-rich repeats per se, is used to measure telomere length. An additional test of this model has monitored the elongation kinetics of a single telomere that is experimentally shortened by an internal recombination event, as described in the previous section (Marcand et al., 1999). Marcand et al. induced shortening by an internal recombination event within the G-rich sequences, thereby leaving the end structure (and terminus-specific regulation) intact. Monitoring of the return to equilibrium length of the shortened telomere revealed that the rate of elongation is inversely proportional to telomere length, such that the rate decreased progressively with increasing telomere length. These results support a model in which the action of telomerase is gradually inhibited, in cis, by the telomere itself, as opposed to the original two-state 'on-off' model for negative regulation of telomerase access. In this model, all of the proteins in the array are counted and the sum determines the dynamics of the telomere. The rate of degradation in the absence of telomerase is constant and independent of starting telomere length, and no steady-state length is achieved (Marcand et al., 1999). Therefore, whereas access of telomerase is limited in a length-dependent manner, access to shortening activities is not.

Blackburn and colleagues have proposed an alternative version of this model, whereby telomere length regulation is predominantly executed by a Rap1p-containing complex assembled onto the distal-most repeats of the telomere, rather than through an array of Rap1 proteins bound along the telomeric tract. This model was proposed on the basis of their investigations in *K. lactis* of the synergistic effects of combining mutations in telomerase and Rap1p. Changes introduced into telomeric DNA that reduce Rap1p binding affinity – by altering the RNA template of *K. lactis* telomerase – resulted in increased telomere length, and the severity of the effect correlated with the degree of loss of Rap1p binding in vitro (Krauskopf et al., 1996). This effect was further enhanced when the *K. lactis* Rap1 protein was truncated at its C-terminus, but reintroduction of wild-type telomeric repeats at the terminus 'capped' the telomere and prevented further elongation. Strikingly, loss of length regulation was observed even when mutant repeat sequences were added only to the very end of the chromosome. These observations lead the authors to propose that the Rap1p complex assembled on the terminal repeats – rather than the array at internal repeats – was crucially required for proper telomere length control. Krauskopf and Blackburn subsequently demonstrated that combining mutations in telomerase and Rap1p accelerated the turnover of telomeric repeats in the distal portion of the telomere (Krauskopf and Blackburn, 1998). It therefore remains unclear as to whether the loss of telomere length regulation is due to loss of Rap1p binding to the terminal repeats, or possibly is a result of changes at the end that provoke an 'autocatalytic' expansion of the array. For example, rapid turnover of telomeric sequences could result in increased telomerase access and/or increased exonuclease action, leading to the observed increased length and heterogeneity. Thus, addition of a few wild-type repeats at the terminus could restore length regulation by returning turnover to wild-type rates and preventing the proposed unregulated balance between lengthening and shortening activities.

NEGATIVE REGULATION OF TELOMERE LENGTH IN MAMMALIAN CELLS: THE TELOMERE FORMS A T-LOOP

Similar lines of investigation in human cells have indicated that proteins complexed with the duplex TTAGGG tract also negatively control telomere length through a cis-acting mechanism. However, the process appears to be more complex,

since two proteins, TRF1 and TRF2, bind to duplex telomeric DNA and act as negative length regulators in human cells. These two proteins share sequence and structural similarity and bind to DNA through Myb-type motifs (Bilaud et al., 1997; Broccoli et al., 1997; Chong et al., 1995). Although both proteins are present at telomeres, TRF1 and TRF2 do not appear to associate with each other (Broccoli et al., 1997), and genetic experiments indicate that each factor makes a distinct contribution to telomere length regulation (see below). Despite the apparent functional similarities between the human and yeast duplex-binding proteins, however, both TRF1 and TRF2 lack significant sequence similarity to the yeast Rap1p protein; furthermore, no homolog of TRF1 or TRF2 has been identified in the sequenced yeast genome.

Experiments examining the consequences of altering the levels of TRF1 and TRF2 have defined a role for these proteins in telomere length regulation. Long-term overexpression of full-length TRF1 shortens telomeres progressively, whereas removal of TRF1 from telomeres results in lengthening (van Steensel and de Lange, 1997). The inhibitory action of TRF1 appears to be mediated through a cis-acting effect on the telomere itself, rather than telomerase expression/activity (van Steensel and de Lange, 1997). Like the yeast Rap1p, TRF1 induces bending of DNA, but it is also capable of looping and pairing duplex DNA in vitro, which argues that it plays an architectural role at the telomere (Bianchi et al., 1997; Bianchi et al., 1999; Griffith et al., 1998). TRF1 might function together with TIN2, a TRF1-interacting protein also implicated in telomere length regulation (Kim et al., 1999). Similar experiments with TRF2 have also uncovered a role for this protein in negative length regulation; like TRF1, increased expression of full-length TRF2 results in telomere shortening (Smogorzewska et al., 2000). Strikingly, however, loss of TRF2 function leads to telomere-telomere fusion events that are a consequence of altered end structure, since telomeres that have diminished TRF2 activity lose their 3′ G strand overhangs. These observations suggest a protective role for TRF2 at the telomere and implicate this duplex-binding protein in the maintenance of the 3′ single-strand overhang.

The recent discovery of a novel conformation that can be assumed by mammalian telomeres has provided a framework for the roles of TRF1 and TRF2 in length regulation and chromosome end protection (Fig. 3). Electron micrographs of psoralen crosslinked murine and human telomere DNA have revealed that the 3′ G-rich single-strand overhang can invade the duplex region of the telomere to generate a lariat structure called a t-loop (Griffith et al., 1999). TRF1 and TRF2 have biochemical activities consistent with formation and/or stabilization of this t-loop structure. TRF1 binds to the duplex telomeric tract and might facilitate loop formation through its DNA-bending properties. TRF2 promotes t-loop formation in vitro and localizes to the junction where the 3′ end invades the duplex tract, which suggests that TRF2 and/or associated proteins aids in strand invasion. This provides a novel model for how induction of a higher-order structure renders a telomere inaccessible to telomerase. Whether the ends of chromosomes in other organisms similarly end in t-loops remains an open question. However, the presence of large duplex loops at the chromosome termini of a ciliated protozoan indicates that this type of end structure is not unique to mammalian chromosomes (Murti and Prescott, 1999).

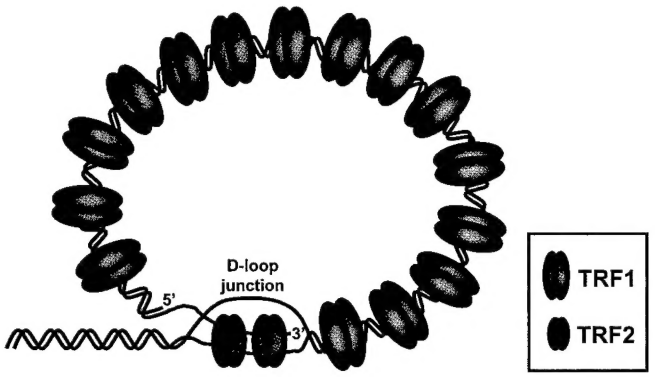

Fig. 3. Mammalian telomeres end in a large duplex t-loop, lariat structure; the 3′ single strand end loops back and invades the duplex region. TRF1 and TRF2 appear to play integral roles in forming and/or stabilizing the t-loop. TRF1 bends, loops and pairs DNA in vitro; these DNA-modeling properties probably aid in forming the architecture of the duplex loop. TRF2 localizes to the D-loop junction at the site of 3′ end invasion in vitro; however, the presence of TRF2 elsewhere along the duplex tract cannot be excluded. Both TRF1 and TRF2 bind to DNA as homodimers (Bianchi et al., 1997; Broccoli et al., 1997). Whether the TRF1-interacting proteins Tankyrase (Smith and de Lange, 1999) and TIN2, and the TRF2-interacting hRAP1 protein play a role in t-loop biology is an interesting question; these proteins are not depicted in this figure.

Although the negative regulatory properties of TRF1 and TRF2 mirror those of Rap1p in the model for yeast telomeres, it is perplexing that neither factor is evolutionarily related to Rap1p. de Lange and co-workers have recently supplied a missing piece of the puzzle by identifying a human TRF2-interacting protein that is the ortholog of budding yeast Rap1p (Li et al., 2000). The human protein, hRAP1, has three domains that have high homology to *S. cerevisiae* Rap1p, including a central Myb-like domain and a C-terminal protein interaction domain. Like its yeast counterpart, hRAP1 is an integral component of telomeric chromatin, and its overexpression results in telomere elongation. However, one key difference distinguishes the budding yeast and mammalian Rap1 proteins: the association of hRAP1 with telomeric chromatin occurs via an interaction with TRF2 – not by direct DNA binding. What contributions hRAP1 makes to the formation of t-loops, and to negative telomere length regulation, remain to be determined.

The identification of a common protein in yeast and humans that negatively regulates telomere length strengthens that idea that both organisms share a mechanism for modulating telomere length. The question that remains, however, is why budding yeast lack TRF proteins. de Lange and colleagues propose that the ancestral telomeric complex consisted of a TRF-like protein that was responsible for DNA binding and also served as a tether to localize a Rap1-type protein. In the budding yeasts, loss of TRF function combined with the ability to bind DNA by Rap1p – perhaps as a result of a divergence in telomeric sequence (i.e. TRF-binding sites) – might have changed the specific players involved but preserved the key components of negative length regulation. This model predicts that Rap1 homologs will be identified in other organisms that possess a TRF-like protein at telomeres, such as fission yeast (Cooper et al., 1997; Li et al., 2000).

PERSPECTIVES

Here we have focused on factors that contribute to telomere homeostasis by regulating, either positively or negatively, accessibility of telomerase to the telomere. Work in both vertebrate and yeast cells is providing a picture of how telomere-associated proteins control whether telomerase gains access to the 3′ terminus. These studies are a keystone required for complete understanding of telomere length regulation; however, many questions remain unanswered. Studies in yeast have suggested that telomere synthesis is restricted to a certain window of the cell cycle (Diede and Gottschling, 1999; Marcand et al., 2000), but the exact stage has not been pinpointed, and the underlying mechanisms are not yet defined. Furthermore, evidence indicates that G strand synthesis by telomerase and C strand synthesis by lagging strand machinery are coupled, but what factors mediate the coordination between the two polymerase machines? What is the role of the human hRAP1 protein in promoting t-loop formation? Do budding yeast chromosomes similarly end in large duplex loops, and, if so, how is this structure generated in the absence of TRF-like proteins – could additional activities possessed by the yeast Rap1p protein, or additional telomere-binding proteins, such as Cdc13p, provide a t-loop-stabilizing function? It is clear that, although the basic players and mechanisms have been defined, the intricate details have yet to be filled in.

Activities that make equally important contributions to maintaining equilibrium telomere length are those that result in telomere shortening through active degradation mechanisms. Factors that regulate these shortening processes are just beginning to be understood. We have discussed Cdc13p in the context of its role in telomerase recruitment, but the end-binding protein also has an essential function at the terminus, because complete loss of Cdc13p function results in rapid resection of one strand of the telomere (Garvik et al., 1995). The Ku heterodimer, initially known for its role in double-strand-break repair, has been postulated to play a separate role in telomeric end protection: loss of Ku protein at the telomere results in a reduced tract length and an altered terminal structure (Boulton and Jackson, 1996; Gravel et al., 1998; Nugent et al., 1998). Although these regulators are being identified, it is still unclear what the end-processing activities are – one candidate is the long-hypothesized exonuclease, possibly the same player that processes double-strand breaks. Thus, many factors impinge on telomere length maintenance. A clear objective in the future will be to determine how all the activities are coordinated to control telomere length regulation.

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